AN ABSTRACT OF THE THESIS OF

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Title: Morphological and Neurological Outcome in the Short Time Study after Spinal Cord Injury in Mice

Abstract approved:

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Spinal cord injury (SCI) is a devastating disease which poses health problems in human and veterinary medicine. SCI causes neurological disability, with loss of motor, sensory and autonomic function. This study investigated the efficacy of local treatment with IKVAV-peptide on spinal cord regeneration following compression injury at T12 vertebra in Balb-c mice. IKVAV-peptide is a membrane spanning peptide known to have a long half-life and the peptide motif IKVAV. Thirty Blab-c female mice were used. Hemilaminectomy was performed at T12 and spinal cords were compressed using extradural application of a 24 g modified aneurysm clip for 1 min in the treatment groups. After 24 hours mice were treated with one of 4 different treatments including isoleucine-lysine-valine-alanine-valaine(IKVAV), IKVAV-peptide, peptide and mannitol (vehicle). Functional improvement was assessed every day using Basso, Beattie, Bresnahan (BBB) Locomotor Rating Scale. 28 days later, the mice were euthanized, and spinal cord segments were studied histologically. Statistical analysis, one-way and two-way analysis of variance (ANOVA) and linear regression model were used to measure some parameters and describe the outcome

after SCI. Over a 4weeks period, IKVAV-peptide group demonstrated statistical and histological evidence of cellular reconstruction and behavioral improvement. The BBB score in the IKVAV-peptide group increased by 5.4 (25%) points, the IKVAV and peptide groups by approximately 1 point (5%) and the mannitol group by 4 points (19%). The number of protoplasmic astrocytes in the IKVAV-peptide group was significantly increased compared to IKVAV, mannitol and normal groups but not with the peptide group ($p<0.001$). Neuron and muscle bundle size were also increased significantly ($p<0.05$ and $p<0.007$, resp.) in the IKVAV-peptide group compared to other treatment groups. The treated control groups showed cellular and gross damages including neuron inactivation and muscle atrophy, gliosis and inability of movement.

Morphological and Neurological Outcome in the Short Time Study after Spinal Cord Injury in Mice

by
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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Soheila Kazemi, Author

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CONTRIBUTION OF AUTHORS

Dr. Mansouri assisted in the writing of chapter 3 and description of the figures.

Dr. Mata and Dr. Karl Schilke were involved with the design and writing of chapter 2.

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DEDICATION

To my Mom, who gives me love and teaches me forgiveness, to my Dad, for his support and courage, to my lovely sister, Roya, who is my best friend

Chapter I

1. Introduction

1.1 Spinal cord anatomy

1.1.1Gross anatomy

The spinal cord is a flattened cylindrical structure which is bilaterally symmetrical. It is enclosed in three membranes or meninges; these are, from without inwards, the dura, arachnoid, and pia matters. Each spinal nerve is joined to its segment of the cord by a number of roots and rootlets grouped as dorsal and ventral roots. Spinal nerve rootlets enter the cord dorsally and leave ventrally. Each spinal nerve is divided into dorsal and ventral branches: the dorsal branch innervates epaxial muscles and ventral branch innervates hypaxial muscles. In cross section, the spinal cord exhibits a butterfly-shaped grayish tan inner substance, the gray matter (roughly in the shape of an “H”) surrounding the central canal, and a whitish peripheral substance, white matter. The central canal is lined by a columnar, ciliated epithelium, the ependym, which is encircled by a zone consisting primarily of neuroglia but also containing a few nerve cells and fibers. During life it contains cerebrospinal fluid. The white matter contains myelinated and unmyelinated ascending and descending tracts (axons), the gray matter contains interneurons and their cell bodies, as well as the cell bodies of the lower motor neurons and neuroglia. Synapses occur only in the gray matter. The gray matter contains a ventral and dorsal horn, and the connection portion that is divided by the central canal into a dorsal and ventral commissure. The ventral

horn contains motor cell bodies that innervate skeletal muscles for reflex and voluntary activity. The dorsal horn contains cell bodies of sensory neurons, pseudounipolar type which bring sensory information from the limbs and organs [1-3].

1.1.2 Functional neuroanatomy

The nervous system along with the endocrine and immune systems and the sensory organs, is responsible for receiving various stimuli and coordinating the reactions of the organism. The stimuli cause impulses that are registered, transmitted, processed and answered in the form of passive or active reactions. Thus, the nervous system enables the body to interact, adapt and react to the environment. A network of nerves connects all organs of the body. This network is comprised of nerve tissue, which can be classified according to function or morphology. This classification is purely didactic; in fact the nervous system acts as a single functional unit. Morphological classification divides the nervous system according to location into central and a peripheral system. The central nervous system (CNS) includes the brain and the spinal cord. Complex systems of neurons are dependent on junctions capable of transmitting nerve impulses between neurons, muscle cells and glandular cells. These gaps between the various cells involved in nerve signaling are termed synapses and play an extremely important role in the transmission of impulses [4]. The function of nervous system is to enable the organism to respond to internal and external stimuli in order to survive. Stimuli are perceived by specific receptors that may be organized

into sense organs (e.g., ear, eye, olfactory mucosa) or dispersed as individual elements. In summary the functional unit of the nervous system is the stimulus-receptor apparatus. It consists of five elements that are outlined here: (1) Receptors and (2) afferent neurons constitute the input limb. (3) The central nervous system ensures adequate processing of the information provided. The output is mediated through (4) efferent neurons and (5) effecter organs. Afferent and efferent elements belong to the peripheral nervous system (PNS) as opposed to the brain and spinal cord, which together form the central nervous system (CNS) or neuraxis.

The physiochemical process of impulse transmission on neurons is always the same regardless of the information being conveyed [5]. There are three typical spinal reflexes which are clinically important including: myotactic reflex, withdrawal reflex and cutaneous trunci reflex [4].

1.2 Spinal cord embryology

The nervous system originates from a dorsal neural tube, and the neural crest, formed from surface ectoderm. The neural tube, whose walls begin as a layer of neuroepithelial cells, becomes the CNS. The neuroepithelial cells then begin a three-phase process of differentiation, which occurs largely in the second month of the fetal development. They proliferate to produce the appropriate number of cells needed for nervous system development. Once they reach characteristic positions in the forming CNS, they differentiate into neuroblasts [6]. The mantle layer becomes the gray matter of the spinal cord, which consists primarily of neuronal cell bodies. Due to differential migrations of immature neurons, the mantle layer becomes shaped like a butterfly with

prominent dorsal and ventral gray columns (horns). Initially the gray columns are uniform along the entire length of the spinal cord. However, as the limbs develop the columns at the corresponding axial levels become significantly enlarged to accommodate neuron cell bodies for each limb. The presence of a greater number of neuronal cell bodies in these regions is the result of degeneration of immature neurons in nonlimb-innervating regions. Most of the cells in regions of the spinal cord without adjacent limbs die because they fail to form viable contacts with developing limb muscle tissue. Also, at the thoracic level (T_1 through L_4), immature neurons migrate dorsomedially out of the ventral motor columns and form the intermediate gray columns. The intermediate gray column contains the neuronal cell bodies of the sympathetic neurons. The marginal layer of the spinal cord is commonly referred to as white matter, so named because of the appearance of myelinated axons. This outer layer contains tracts of ascending (projecting cranially) and descending (projecting caudally) axons that are grouped together in bundles called funiculi [7].

Approximately 100 billion neurons are present in the CNS, including the interneurons and the perikaryons of the motor neurons from the body's cerebrospinal (somatic) nerve system. The neurons are highly specialized structurally and functionally and have lost their ability to divide. New neurons develop from precursor cells, the neuroblasts only. Once a neuron is lost, it can not be replaced. With intensive training, the loss of neuronal function due to small injuries cannot be fully regained by neogenesis of the original neuron network but some function can be return using collateral neurons [4].

1.3 Spinal cord histology

1.3.1 Normal spinal cord histology

A basic function of the nervous system is communication. The central nervous system (CNS) consists of the brain and spinal cord and contains the nerve cells, or neurons, and a host of supporting cells called the neuroglia [8]. Neuroglia provide protection for delicate neuronal processes, electrical insulation for nerve cell bodies and their processes and metabolic exchange pathways between the vascular system and the neurons of the nervous system [1]. Nerve impulses pass to and from the CNS over long neuronal processes called axons. The peripheral nervous system (PNS) comprises all of these processes which travel in cranial and spinal nerves and related clusters of outlying neurons known as ganglia. Neurons exhibit an astonishing variety of forms, but each has a cell body, or soma, consisting of a nucleus and its surrounding cytoplasm, the perikaryon [8]. The cell body of a neuron has characteristics of a protein- producing cell. The cytoplasm reveals abundant rough endoplasmic reticulum (RER) and free ribosomes when observed with the transmission electron microscope (TEM). At the light microscopic level, the basophilic materials which consist of free ribosomes and RER appear as small bodies, Nissl bodies. The cytoplasm also contains numerous mitochondria, a large perinuclear Golgi apparatus, lysosomes, microtubules, neurofilaments, transport vesicles, and inclusions. The neuron cell body usually has several radiating processes called dendrites and a single long slender process, the

axon. As expected of cells active in protein synthesis, the Golgi apparatus is prominent in all neurons. In electron micrographs, Golgi (a loose network of wavy strands) is seen as flat cisternae, often slightly dilated at their ends. It concentrates and slightly modifies proteins synthesized in the granular endoplasmic reticulum. The motor neuron, together with the muscle fibers it innervates, is called a motor unit. Each motor nerve fiber branches to supply many muscle fibers. It is generally accepted that nerve cells do not divide; however in some areas of the brain neural stem cells are present and are able to differentiate and replace damaged nerve cells [1].

1.3.2 Neuroglia

These are not merely mechanically supportive cells but are metabolically active elements that assist nerve cells in performing their integrative and communicative functions. These cells consist of astrocytes, oligodendrocytes, and microgliocytes. Unlike neurons, glial cells do not seem to play a direct role in neural communications. They respond passively to electrical currents and do not generate impulses [8].

1.3.3 Astrocytes

The star-shaped glial cells called astrocytes which are classified into two types, protoplasmic and fibrous. These cells provide physical and metabolic support for the neurons of the CNS. Protoplasmic astrocytes, which are found mainly in the gray matter of the CNS, have numerous, short, branching cytoplasmic processes. Fibrous astrocytes are found mainly in the white matter but also in some regions of the

preventricular gray matter. They have long thin processes that branch infrequently. Astrocytes have an important role in homeostasis in the CNS. Astrocytes are closely associated with neurons to support and modulate their activities. It is now thought that astrocytes play a role in the movement of metabolites and wastes to and from neurons and regulate ionic concentrations in the intercellular compartment, thus maintaining the microenvironment and modulating activities of the neurons. They also have a role in maintaining tight junctions of the capillaries that form the blood-brain barrier. In addition, astrocytes provide a covering for the "bare areas" of myelinated axons, e.g., at the nodes of Ranvier and at synapses, but they do not form myelin. They may confine neurotransmitters to the synaptic cleft and may remove excess neurotransmitters by pinocytosis. Protoplasmic astrocytes at the brain and spinal cord surfaces extend their processes to the basal lamina of the pia mater to form the glia limitans, a relatively impermeable barrier surrounding the CNS. Ependymal cells form the epithelium-like lining of the fluid-filled cavities in the CNS. They form a single layer of cuboidal-to-columnar cells that have the morphological characteristics of fluid-transporting cells [1, 8].

1.3.4 Histopathology of the spinal cord following trauma

Severe injury to the CNS commonly results in permanent, crippling deficits for the remainder of the patient's life. The apparent inability of the CNS to regenerate and reconstitute fiber tracts that have been interrupted (in contrast to the reasonable efficiency with which this can occur in the peripheral system) has both fascinated and

frustrated clinicians and investigators for decades. It appears that the milieu of the brain and spinal cord is inhibitory to axonal regrowth; whereas the same nerve fibers extend perfectly well if surgically directed into PNS environment through using the scaffold. Astrocytes have previously been considered one element antagonistic to CNS repair, but recent investigations point to myelin membrane proteins as important sources of neurite growth inhibitions. In the presence of monoclonal antibodies that neutralize these proteins or in newborn rats which are irradiated to produce a zone of hypomyelination, central axonal regrowth can occur. The injuries to the brain and spinal cord produced by trauma vary considerably, depending upon three factors: the severity, speed and duration. Acute, explosive injury, as with a sudden intervertebral disk prolapse, may produce hemorrhage, edema and ischemia; gray matter is more sensitive to such injuries than is white matter. If the spinal cord is acutely damaged, leukocytes (mainly neutrophils) enter the damaged tissue in response to chemotactic signals within 3 to 6 hours. Blood monocyte-derived mononuclear macrophages are evident by the third day, and the neutrophilic influx wans by day 5. Cell death in the gray matter may be evident by 4 hours, but the area if necrosis progressively expands for a few days. In some cases, the whole spinal cord is necrotic, maintained in situ by a meningeal sleeve. The tissue liquefies, becoming soft and paste-like. Less dramatic lesions produce central cystic cavities. From the level of the spinal cord injury, a core of necrosis may extend cranially and caudally, typically in the base of the dorsal funiculi. Isolated areas of necrotic tissue, removed from the immediate point of impact, probably reflect regions of ischemia following post-traumatic secondary injury

to blood vessels. Chromatolysis of ventral horn neurons occurs as a response to injury to the proximal segment of their axons and is called the axonal reaction. Reactive astrocytes may contain immunoglobulins, apparently sequestered following blood-brain barrier disruption.

Acute, traumatic spinal cord injury is mediated by primary and secondary mechanisms. The primary event is the mechanical injury to the tissue, which may include compression, distraction and laceration. Secondary changes are the interruption to normal vascular perfusion, as well as such factors as electrolyte shifts, excitotoxic mechanism of neuronal damage and free radical damage to cells and DNA. There is experimental evidence of very early axoplasmic and myelin changes, which may reflect calcium-mediated events that precede vascular insufficiency. A cascade of further mediators of tissue degeneration which appears later includes: leukocyte products, prostaglandins, leukotrienes, free radicals, and excitotoxins. This has prompted the use of variety of pharmacological antagonists in attempts to improve the management of acute spinal cord trauma. Acute spinal cord injury results in a significant reduction in spinal cord blood flow, which contributes to post-traumatic ischemia [9].

1.4 Neurophysiology

Neurons are highly responsive to stimuli. This response is called the action potential (nerve impulse) and it underlies virtually all functional activities of the nervous system [6].

1.4.1 Normal cell populations and their roles in the spinal cord

We often think of the spinal cord as being only the conduit for signals from the periphery of the body to the brain or in the opposite direction from the brain back to the body. But the spinal cord has many other highly organized functions, for instance, neuronal circuits in the cord can cause: walking movements, reflexes that withdraw portions of the body from painful objects, reflexes that stiffen the legs to support the body against gravity, and reflexes that control local blood vessels, gastrointestinal movements, or urinary excretion [10]. The nervous system is the master controlling and communicating system of the body [6]. The billions of neurons, also called nerve cells, are the structural units of the nervous system. They are highly specialized cells that conduct messages in the form of nerve impulses from one part of the body to another. Besides their ability to conduct nerve impulses, neurons have some other special characteristics. They have extreme longevity. Given good nutrition, neurons can function optimally for a lifetime (over 100 years). They are largely amitotic. As neurons assume their roles as communicating links of the nervous system, they lose their ability to divide. We pay a high price for this neuron feature because they can not replace themselves if destroyed. They have an exceptionally high metabolic rate and require continuous and abundant supplies of oxygen and glucose. Neurons can not survive for more than a few minutes without oxygen [6]. Glia, are important in the normal functioning of the nervous system. For example, glia are involved in potassium

ion buffering; they provide pathways for neuronal migration during development and for axonal guidance during regeneration. They release cytokines in disease processes and proliferate or hypertrophy following injury. While they do not participate directly in the propagation of nerve impulses, they are involved in Ca ion buffering and neurotransmitter reuptake. As a result of injury, astrocytes tend to enlarge and proliferate. This process is known as gliosis and results in a glial scar [11].

1.4.2 Spinal cord injury

The spinal cord is the major pathway through which nervous signals pass between the brain and the body. Therefore spinal cord injury (SCI) results in an interruption of these pathways and loss of many bodily functions [12]. SCI, results from sudden or sustained trauma or progressive neurodegeneration [13].

Since traumatic injury of the central nervous system results in formation of scar tissue and axon-growth inhibitory molecules, SCI was believed to be irremediable [12, 14]. However, extensive basic and clinical research during the past years have demonstrated complex processes underlying the SCI which has provided the basic information for developing the promising therapies for this devastating disease [12, 15].

1.4.2.1 Spinal cord response to injury

Some tissues of the body retain the ability to form new cells from existing precursors during the life of the animal, but the nervous system is severely limited in

this respect. In general, there is very little generation of new nerve cells once development is completed. This is, of course, the main reason why injuries to the nervous system have such devastating effects [11].

1.4.2.2 Response of neurons to injury

1-Degeneration, which means that the portion of a nerve fiber distal to a site of injury degenerates because of interrupted axonal transport. The cell body of an injured nerve swells, its nucleus moves peripherally, and there is loss of Nissl substances, called chromatolysis. 2-Scar tissue formation which derives from proliferating glial cells that are considered to prevent regeneration [1].

1.4.3 Spinal cord injury at molecular and cellular level

Prevention of SCI and treatment of SCI disabilities are goals that can be reached by understanding the pathophysiological changes after injury [16]. Currently, the pathophysiology of acute SCI is separated into primary and secondary mechanisms [17]. After mechanical damage (primary mechanism) to the spinal cord which leads to immediate hemorrhage and rapid cell death, a secondary cascade occurs. This cascade causes multiple reactions within the body including ischemia, disarrangement of ion concentrations, excitotoxicity, reactive oxygen species, inflammation, apoptosis and scar formation over weeks to months [18, 19]. The biological response to a spinal cord injury is branched into three phases that follow a definite but partially overlapping temporal sequence: acute (seconds to minutes after the injury), secondary (minutes to weeks after the injury), and chronic (months to years after the injury) [19].

1.4.4 Spinal cord injury: Primary and secondary physiological effects of trauma to the spinal cord

1.4.4.1 Acute phase

In the acute phase, primary damage occurs as a direct result of trauma such as contusion, compression, or laceration leading to immediate physical and biochemical cellular alterations and marked by systemic as well as local events. These include systemic hypotension, spinal shock, vasospasm, ischemia, plasma membrane compromise, derangements in ionic homeostasis, and accumulation of neurotransmitters [17, 20]. The primary injury damages both upper and lower motor neurons and disrupts motor, sensory and autonomic (including cardiac output, vascular tone, and respiration) functions [13].

1.4.4.2 Secondary phase

Pathophysiology processes occurring in the secondary injury phase are rapidly influenced in response to the primary injury in an attempt to homeostatically control and minimize the damage. These processes are paradoxically and largely responsible for exacerbating the initial damage and creating an inhibitory milieu, which prevents endogenous efforts of repair, regeneration, and remyelination [13]. Great effort has been made to understand the pathophysiological changes underlying SCI in the hope of developing neuroprotective strategies and preventing disability. Despite its heterogeneous causes, SCI evolves into secondary damage affecting apparently spared areas, magnifying the disability and amplifying neurodegeneration. The challenge is to clarify why and when such damage occurs. SCI causes not only neuronal and

oligodendroglial cell death, but also induces microglial-associated inflammatory responses and reactive astrogliosis. The latter alterations contribute to tissue loss and glial scar formation. A key mechanism responsible for secondary injury after trauma is increased microglial proliferation and associated activation reflected by production of pro-inflammatory cytokines and neurotoxic molecules. In parallel, astrocytes move away from the center of the lesion, become hypertrophic, proliferate, and up-regulate the expression of Glial Fibrous Acidic Protein (GFAP). Hypertrophic astrocytes are the major cellular component of the glial scar, which is considered a physical and molecular barrier to CNS regeneration. Moreover, reactive astrocytes also produce several classes of growth inhibitory molecules, including the family of extracellular matrix molecules, known as chondroitin sulphate proteoglycans, which inhibit both in vitro and in vivo axonal regeneration. Considerable evidence indicates that preventing and/or reducing the inhibitory environment of the glial scar provides a better environment for neurons to regenerate. Current knowledge on the role of cell cycle events in SCI is derived mainly from rodent SCI models. Up-regulation of cell cycle-related proteins occurs in both neurons and glia after SCI may contribute to secondary damage cascades [21]. The secondary injury is a cascade of cellular and molecular events that intensify the initial damage. The secondary phase features a continuity of some events from the acute phase –disruption of vasculature and increased permeability of the blood-spinal cord barrier, electrolyte shifts, edema, and necrotic cell death - as well as novel ones, including the formation of free radicals, delayed calcium influx, immune system response or and inflammation, and apoptotic cell

death. These mechanisms are interconnected in a self- propagating cycle that perpetuates each other once initiated by trauma [20, 22]. The secondary injury starts minutes after primary insult and can lasts up to weeks after injury. Extracellular glutamate levels are known to increase transiently within the first 3 h after SCI, with a likely second wave of glutamate release 2–3 days after injury, probably due to delayed myelin destruction that compromises nearby axon integrity. The early phase comprises vasogenic and cytotoxic edema, necrosis, excitotoxicity, early demyelination, and systemic events like hypotension (2–48 h). Later, macrophage infiltration and initiation of glial scar occur. Within 2 weeks/6 months, glial scarring continues together with intraspinal cyst formation. Even later, profound pathological changes affect spinal networks through Wallerian degeneration, demyelination, and aberrant plasticity with circuit rewiring leading to dysfunction like chronic pain and spasticity. The molecular cell-death pathways of SCI (and their effectors) remain largely elusive. This condition makes it difficult to identify the best time window for satisfactory treatment of acute SCI with the aim of limiting (or even preventing) secondary damage. Nonetheless, the consensus is that the time to introduce effective neuroprotective strategies after SCI is short, probably restricted to the first hours after injury, in analogy with a similar situation for the brain. Studies of brain and spinal injuries support the theory that the central nervous system (CNS) responds to a lesion in an archetypal fashion, regardless of the insult, and that similar pathological pathways and cell-death mechanisms may operate in the brain and spinal cord [23-28].

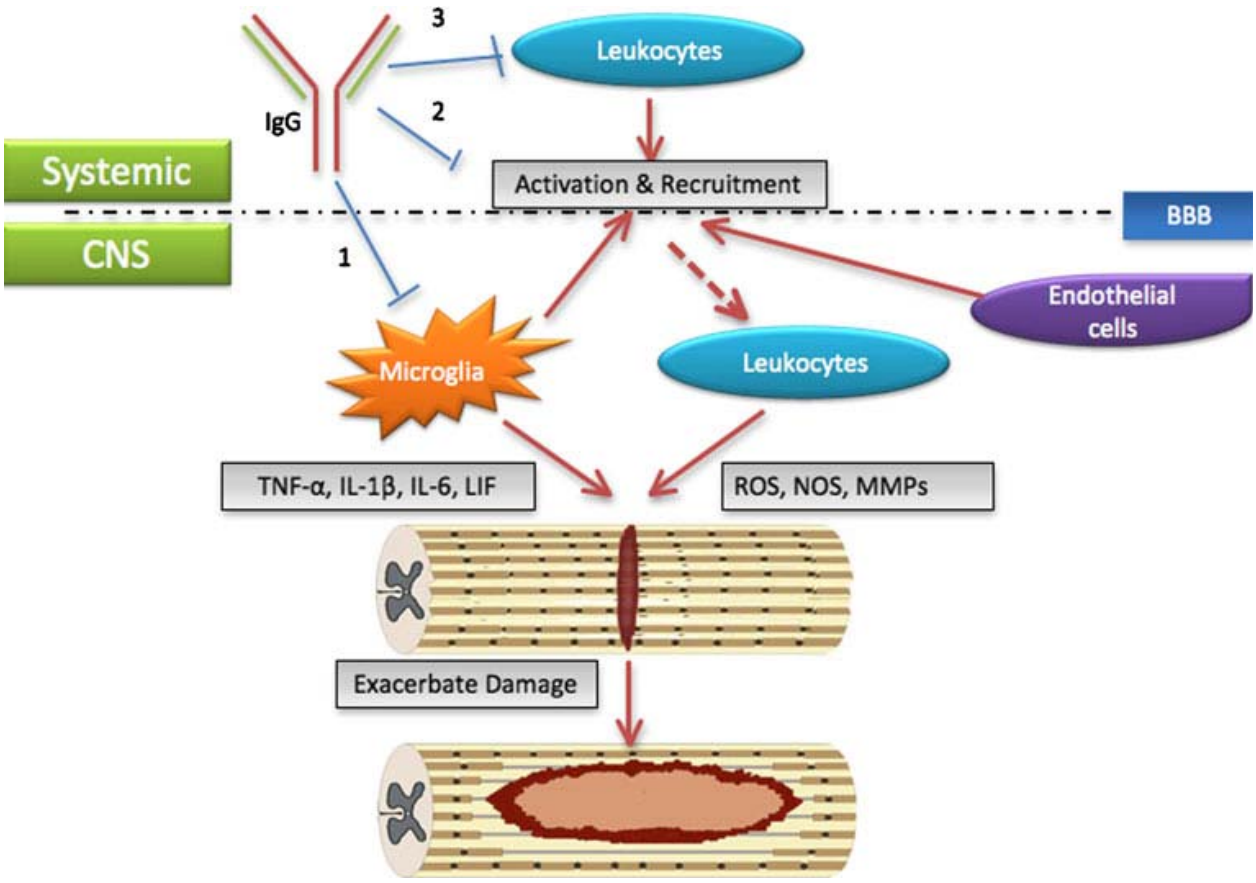

Figure 1.1. Schematic view of potential interactions of IgG with the neuro inflammatory response following SCI.

The above schematic diagram represents how the infiltration of microglia and leukocytes could exacerbate the initial damage. Microglia and leukocytes are recruited to the injury epicenter where they produce neurotoxic substances that cause neuronal and oligodendrocytic cell death in the secondary injury cascade. IgG has the potential to reduce the extent of secondary damage by interacting with microglia and leukocytes in the following mechanism: (1) inhibit microglia activation and reduce pro-

inflammatory cytokine production, (2) inhibit leukocyte recruitment to the injury epicenter following SCI by acting on endothelial cells, and (3) inhibit leukocyte activation by upregulation of the inhibitory FcγIIB receptor. BBB (blood-brain barrier), LIF (leukemia inhibitory factor), ROS (reactive oxygen species), NOS (nitrous oxide synthase), MMPs (matrix-metalloproteinases) [23]. The end result of the primary and secondary mechanism events is the considerable loss of cells, cavitations, and the formation of a glial scar [13].

1.4.4.3 Inflammatory response

The inflammatory response is crucial for the clearance of cellular debris, but inflammatory molecules and the inflammatory cascade might contribute to inhibition of regeneration of surviving neurons. In addition, over activation of the inflammatory response can destroy normal tissue which can result in further loss of function following SCI [23]. In the injured spinal cord, microglial cells, immediately after injury, secrete pro-inflammatory cytokines, including interleukin (IL)-1β, interleukin-6, and tumor necrosis factor-α (TNF-α), all of which increase the extent of inflammation. However, in the acute phase of SCI, immune reactive cells can damage healthy tissues by releasing pro-inflammatory cytokines, reactive oxygen species (ROS), and matrix-metalloproteinases [29-31]. In addition to pro-inflammatory cytokines, the injured site also has anti-inflammatory cytokine transforming growth factor-β (TGFβ). The treatment of SCI rats with TGF-beta reduces lesion volume by 50% at 48 h and this is associated with decreased accumulation of mononuclear

phagocytes in and around the injury site. This reduction of mononuclear phagocyte numbers around the site of trauma would reduce their contribution to secondary injury [32].

1.5 Spinal cord injury complications

1.5.1 Vascular mechanisms and complications

Identification of cardiovascular complications after acute SCI is essential to minimize adverse outcomes and to optimize recovery [33]. Normal spinal cord is required for suitable autonomic control of different viscera and organs including the heart and blood vessels [33]. The role of the vascular mechanisms which have been obtained from many different angiography methods from a variety of models of the acute SCI in several species support the theory and the hypothesis that posttraumatic ischemia is a key mechanism and important secondary mechanism of SCI [34]. Major cardiovascular complications in the acute stage following SCI include, orthostatic hypotension, heart rate abnormalities and venous thromboembolism [33, 35-38].

1.5.2 Urinary complications

Control of the lower urinary tract (LUT) is a coordination of the urinary bladder and urethral sphincter, which both are under the control of peripheral and different levels of the central nervous system. Injuries and diseases such as SCI affect the neuronal regulation of LUT by interruption of ascending and descending connection fibers between spinal cord and supra spinal center. In most cases, SCI

results in a period of bladder areflexia and dysfunction followed by emergence of neurogenic detrusor over reactivity [39, 40].

1.5.3 Muscle dysfunction

It has generally been accepted that muscles that are paralyzed as a result of spinal cord injuries undergo atrophy and develop less force. Muscle atrophy, a reduction in the size and/or number of muscle fibers, may be present as denervation atrophy or disuse atrophy [41]. Denervation atrophy is an outcome from injury to motor neurons of the spinal cord or to the motor nerves in the ventral roots through which they exit. Disuse atrophy is a result of loss of muscle activation due to disruption to the central and segmental synaptic drive onto the surviving spinal motor neurons which occurs during most SCI in humans [42].

1.5.4 Gastrointestinal complications

Bowel problems occur in 27% to 62% of patients with spinal cord injuries (SCI), most commonly constipation, distention, abdominal pain, rectal bleeding, hemorrhoids, bowel accidents, and autonomic hyperreflexia. The acute abdomen, with a mortality of 9.5%, does not present with rigidity or absent bowel sounds but rather with dull/poorly-localized pain, vomiting, or restlessness, with tenderness, fever, and leukocytosis in up to 50% of patients. Fecal impaction may present with anorexia and nausea [43].

1.6 Incidence of spinal cord injury

According to a study initiated by the Christopher & Dana Reeve Foundation, approximately 1.9% of the U.S. population, or some 5,596,000 people, reported some form of paralysis based on the functional definition used in the survey. Approximately 0.4% of the U.S. population or some 1,275,000 people reported being paralyzed due to a spinal cord injury. It means that we all know someone -- a brother, sister, friend, neighbor, or colleague -- living with paralysis [44-46]. Approximately 12,000 new cases in the USA are reported each year, and the average medical cost for a patient with SCI can be up to $10 million. Besides the financial charge, patients with SCI and their relatives are involved with the physical, emotional, and social effects of this debilitating condition [23]. Although many studies are focusing on different molecular mechanisms to prevent further problem and dysfunction following SCI as well as methods to improve recovery following SCI, there is no evidence that the incidence of SCI is declining [34].

1.7 Targets for intervention in spinal cord injury

Targets of therapies for SCI to improve function can be simplified into a list: *1*) reduction of edema and free radical production, *2*) rescue of neural tissue at risk of dying in secondary processes such as damage by abnormally high extracellular glutamate concentrations, *3*) control of inflammation by anti-inflammatory agents, *4*) rescue of neuronal/glial populations at risk of continued apoptosis, *5*) repair of demyelination and conduction deficits; *6*) promotion of neurite growth through

improved extracellular environment, *7*) cell replacement therapies, *8*) efforts to bridge the gap with transplantation approaches, *9*) efforts to retrain and relearn motor tasks, *10*) restoration of lost function by electrical stimulation, such as bladder and bowel function or hand function, retrain the brain by aggressive physical therapy and *11*) relief of chronic pain syndromes [18].

1.8 Treatment of traumatic spinal cord injury

Despite advances in pre-hospital care, medical and surgical management, and rehabilitation approaches, many SCI sufferers still experience substantial neurological disability [13]. Cell therapy is a research area to assess the nature of the pathophysiology of secondary SCI. Different approaches using different types of cells have been studied for many years, including stem cells and non-stem cells. Stem cell therapy contains cell sources such as embryonic stem cells, neural progenitor cells, bone marrow mesenchymal cells. Non-stem cells include olfactory ensheathing cells and Schwann cells [13, 47]. Cellular transplantation after SCI has several aims: to bridge any cysts or cavities; to replace dead cells (for example, to provide new neurons or myelinating cells); and to create a favorable environment for axon regeneration. Cellular therapeutic interventions include: transplantation of peripheral nerves, transplantation of Schwan cells, transplantation of olfactory nervous system cells, transplantation of embryonic CNS tissue, transplantation of embryonic stem/progenitor cells, transplantation of adult stem/progenitor cells, transplantation of engineered stem/progenitor cells, transplantation of activated macrophages and

transplantation of stem cell-derived oligodendrocyte progenitor cells [48, 49]. Molecular therapies after SCI have several aims: to protect neurons from secondary cell death; to promote axonal growth; and to enhance conduction. Molecular therapeutic interventions include: neuroprotective therapies, therapies that enhance remyelination or enhance conduction such as potassium channel blocker that can improve axonal conduction, delivery of growth factors, such as brain-derived neurotrophic factor (BDNF), and basic fibroblast growth factor, delivery of cAMP or small GTPases. Researchers have also investigated modulation of interactions with myelin inhibitors and extracellular matrix modifiers [48].

Pharmacological treatments also have been one of the methods in this area, their strategies focus on the control of secondary injury processes. This control primarily includes lipid peroxidation (LP), the salvage of as much white matter as possible and suppression of fibrous scarring by local inhibition of collagen biosynthesis and basement membrane deposition [14, 50].

Nanomedicine and biomaterial design strategies have been devised to promote axonal extension through a site of injury and provide the extracellular environment to prevent the inhibitory molecules during the acute and chronic phase of SCI. There are a variety of nanostructured scaffolds which can make an environment to enhance the chance of axon elongation and neuron regeneration. Many experts agree that the greatest hope for treatment of spinal cord injuries will involve an approach that integrates biomaterial scaffolds, cell transplantation, and molecule delivery. Some

biomaterial strategies for spinal cord injuries include silicone, chitosan and collagen [51]. Peptide amphiphiles (PA) are synthetic molecules that self- assemble into nanofibers [52]. A family of PAs has been demonstrated to self-assemble reversibly into a nanofiber network, which result in the formation of aqueous gels through pH changes. The PA fibers can then be reversibly polymerized to enhance their stability. These two switchable events controlling the formation of supramolecular structure and polymerization produce a remarkably versatile material [53]. Few treatments are currently available or are even being investigated in clinical trials that address neurological impairment following traumatic SCI [54]. Therefore, there is a dire need for effective treatments that can reduce neurological deficit and improve a patient's quality of life following SCI. Unfortunately despite all the efforts have been done so far in this area, no satisfactory therapy is currently available [55].

1.9 IKVAV-peptide

1.9.1 IKVAV, its function and role in the spinal cord

Self-assembling nanofibers are bioactive and biocompatible materials which are used in neuronal tissue engineering [56]. Laminin is a major basement membrane-specific glycoprotein [57]. So far, five alpha, three beta, and three gamma chains have been identified, and at least 15 isoforms are formed by various combinations of each subunit. Laminin-1, consisting of three chains designated alpha 1, beta1, and gama1, has diverse biological activities. Several active sequences of laminin-1 have been identified using synthetic peptide approaches. An Ile-Lys-Val-Ala-Val (IKVAV)

sequence located on the C-terminal end of the long arm of the alpha1 chain promotes cell adhesion, neurite outgrowth, angiogenesis, collagenase IV production, and tumor growth [58]. It has been shown that some of the analogs, in which the lysine and isoleucine residues of the IKVAV peptide were substituted with different amino acids, promoted cell attachment, but none of the analog peptides showed neurite outgrowth activity comparable to that of the IKVAV peptide. These results suggest that the lysine and isoleucine residues are critical for the biological functions of the IKVAV peptide [59]. Tube formation is a multi-step process induced by laminin. An RGD-containing sequence in the A chain of laminin through an integrin receptor on the endothelial cell induces their attachment to the protein while a YIGSR site in the B1 chain induces cell-cell interactions and the resulting tube formation. Also the laminin-derived synthetic peptide YIGSR contains sufficient information to induce single endothelial cells to form ring-like structures surrounding a hollow lumen, the basic putative unit in the formation of capillaries [60]. Tumor cells attach, degrade, and migrate through basement membranes as they metastasize. Laminin, a major glycoprotein of basement membranes, promotes the metastatic activity of tumor cells by stimulating the attachment and migration of the cells and their secretion of collagenase IV. A synthetic peptide of 19 amino acids (Cys-Ser-Arg-Ala-Arg-Lys-Gln-Ala-Ala-Ser-Ile-Lys-Val-Ala-Val-Ser-Ala-Asp -Arg) from the sequence of the A chain of laminin increases experimental metastases of the lungs by murine melanoma cells. The peptide is active when injected either intravenously or intraperitoneally. The peptide increased collagenase IV activity, a key enzyme in the breakdown of basement membranes, to

the same extent as laminin. This peptide represents an active site on laminin for promotion of the metastatic phenotype and generates a probe for studying the regulation of malignant activities [61]. Neurons from peripheral and central nervous tissue as well as from established cell lines respond to low concentrations of laminin with rapid extension of axon-like processes. Two sites on laminin have been identified which stimulate neurite outgrowth, the major site residing at the end of the long arm of laminin. Recently laminin has been cloned and sequenced allowing for synthetic peptides to be prepared and tested for biological activity. Antisera against synthetic peptides corresponding to A and to B1 chain sequences at the end of the long arm can partially inhibit laminin-mediated neurite outgrowth. A 19 amino acid synthetic peptide (CSRARKQAASIKVAVSADR) from the long arm of the laminin A chain is capable of stimulating neuronal-like process formation to almost the same extent as laminin and competes with laminin for stimulation of neurite outgrowth [62]. A 19-mer peptide (designated PA22-2), from just above the carboxyl globule on the long arm of the A chain, was found to promote cell adhesion, spreading, migration, and neurite outgrowth. By testing smaller sequences within the 19-mer peptide, a constituent pentapeptide, IKVAV (Ile-Lys-Val-Ala-Val), was identified as the active site for cell adhesion and neurite outgrowth. These data suggest that this sequence is one of the principle sites in laminin which regulate cellular behavior [63]. Peptide amphiphile molecules are designed to form cylindrical nanofibers that display to cells in the spinal cord the laminin epitope IKVAV (isolucine-lysine-valine-alanine-valine) at nearly van der Waals density. The nanofibers containing the IKVAV epitope have

been demonstrated to promote regeneration of both descending motor fibers and ascending sensory fibers through the lesion site. These nanofibers also resulted in significant behavioral improvement, reduced cell death, increased number of oligodendrocytes and reduced astrogliosis [64]. Self-assembling peptide scaffolds consist of alternative amino acids that contain 50% charged residues. These peptides are characterized by their periodic repetition of alternating ionic hydrophilic and hydrophobic amino acids that spontaneously form beta sheet structures. These beta sheets have distinct polar and non-polar surfaces. They are also buoyant in aqueous solution and readily transportable to different environments. Upon exposure to aqueous solution with neutral pH, ions screen the charged peptide residues and alanines (which form the non-polar surfaces of the beta sheets) of different beta sheets pack together as a result of their hydrophobic interactions in water. This gives rise to double-layered beta sheet nanofibers, a structure that is found in silk fibroin from silkworm and spiders. Thus the final self-assembly step to create the peptide scaffold takes place under physiological conditions [65]. The nanofibers that self-assemble in aqueous media place the bioactive epitope on their surfaces at van der Waals packing distances. These nanofibers bundle to form 3D network and produce a gel-like solid. The nanofibers have high aspect ratio and high surface areas, 5 to 8 nm in diameter and width length of hundreds of nanometers to a few micrometers. Thus, the nanofibers that form around cells in 3D present the epitopes at an artificially high density relative to a natural extracellular matrix [66].

1.9.2 Transmembrane spanning peptides

Membrane proteins form key nodes in mediating the cell's interaction with the surroundings, which is one of the main reasons why the majority of drug targets are membrane proteins [67]. Membrane-spanning domains are composed mainly of alpha helices, whose associations within the cellular membrane are generally governed by electrostatic and van der Waals interactions [68]. Helix-helix interactions are important for the folding, stability, and function of membrane proteins [69]. α-Helical membrane proteins fulfill many vital roles in all living cells and constitute the majority of drug targets [70]. They play numerous and diverse functions, including water and solute transport, signal transduction and integration, enzymatic activity, and anchorage of cytoskeletal or extracellular components. Membrane proteins account for about one third of all proteins encoded in the human genome and make up more than half of all current drug targets [71, 72]. Rationally designed nanomedicines have the potential to overcome obstacles to tissue regeneration by allowing presentation of bioactive molecules for longer periods while guiding the regenerative processes. We incorporated the laminin-derived bioactive sequence, IKVAV, to the cell-spanning peptide to increases the half-life of one or more bioactive molecules within the site of injury may improve regeneration of nerve tissue following spinal cord injury (SCI).

1.9.3 IKVAV-peptide for treatment of spinal cord injury

Peptide amphiphile (PA) molecules that self-assemble from aqueous solution into cylindrical nanofibers that display bioactive epitopes on their surfaces were used

as a therapy in a mouse model of spinal cord injury [64]. IKVAV PA (a negatively charged PA incorporating the neuroactive pentapeptide epitope from laminin, isoleucine-lysine-valine-alanine-valine (IKVAV)) nanofibers are known to inhibit glial differentiation of cultured neural stem cells and to promote neurite outgrowth from cultured neurons. I*n vivo* treatment with the PA after SCI reduces astrogliosis, cell death, and increases the number of oligodendroglia at the site of injury. Furthermore, the nanofibers promote regeneration of both descending motor fibers and ascending sensory fibers through the lesion site. Treatment with the PA also causes significant behavioral improvement. These observations demonstrate that it is possible to inhibit glial scar formation and to facilitate regeneration after SCI using bioactive three-dimensional nanostructures displaying high densities of neuroactive epitopes on their surfaces [64].

IKVAV-peptide used in this experiment is a membrane spanning peptide expressing the IKVAV epitope on spinal cord. With this method of administration, the neurotrophic motif can be presented without the use of extraneous materials such as gelatin or nanofibers. Furthermore, our previous work has demonstrated that once injected into tissue, the peptide remains at the site of the injection with a long half-life. This assured us that these peptides could provide a sustained signal within the lamina to enhance repair of neurons and restore function to the injured spinal cord. Regeneration of nerve function can be stimulated by proper presentation of the neurite stimulating IKVAV-peptide at the site of the injury [64].

1.10 Statement of objectives and investigational rationale

1.10.1 Objectives

The goal of the project was to demonstrate that the peptide IKVAV conjugated with a membrane spanning peptide acts by influencing the spinal cord's response to injury. Further, the research determined the effects and safety of the IKVAV-peptide when injected into the site of injury in mice. The final objective was to document the effects of IKVAV-peptide on neurons and astrocytes at the site of spinal cord injury and on peripheral skeletal muscle following spinal cord injury in mice. Finally, the effects of a single injection of IKVAV-peptide were determined by blinded observation of injured mice and measurement of their BBB scores at 28 days following injury.

1.10.2 Rationale

In the present study, IKVAV-peptide treated mice revealed the largest number and size of active neurons compared with all other treatment groups ($p<0.05$), thus indicating that the IKVAV-peptide may induce a progressive, active phase in neurons to stimulate normal function and even regeneration. However, in the other treatment groups, reduction of neuron size characterized with shrunken cell cytoplasm may have represented necrotic and apoptotic phases of SCI. The rationale for use of the IKVAV motif is based upon its ability to stimulate neuron regeneration. By attaching this peptide to a membrane spanning peptide we hypothesized that the sustained

presentation of IKVAV following injury would improve functional recovery following spinal cord injury.

Chapter II

A cell spanning IKVAV expressing peptide for treatment of spinal cord injury

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Abstract

Efficacy of local treatment with a membrane spanning peptide expressing the isoleucine-lysine-valine-alanine-valine (IKVAV) epitope on spinal cord regeneration was assessed following compression injury in Balb-c mice. The day after hemilaminectomy and compression injury, mice were treated with one of the following: IKVAV, IKVAV-peptide, peptide or mannitol/saline (vehicle). Functional improvement in movement was assessed daily and spinal cord segments were studied histologically 28 days after injury. The Basso, Beattie, and Bresnahan (BBB) score for locomotion in the IKVAV-peptide group increased by 5.4 (25%) points, the IKVAV and peptide groups by approximately 1 point (5%) and the mannitol group by 4 points (19%). The number of protoplasmic astrocytes in the IKVAV-peptide group was significantly increased compared to IKVAV, mannitol, and normal groups but not with the peptide group ($p<0.001$). Neuron and muscle bundle size were also increased significantly ($p<0.05$ and $p<0.007$, resp.) in the IKVAV-peptide group compared to other treatment groups. The observations in this study demonstrated that it is possible to promote functional recovery after spinal cord injury (SCI) using bioactive IKVAV presenting cell spanning peptides.

Background

Cell penetrating peptides designed to deliver drugs and respond to changes in pH were first reported by Summerton et al. [73]. Later versions of this approach to drug delivery have included pH-sensitive membrane peptides (pHLIPs) derived from

the bacterioehodopsin C helix [74] and rationally designed pH activated peptides [75]. These technologies have generally been applied to delivery of macromolecules into tumor cells. However, these molecules have potential for enhancing the delivery and increasing the efficacy of biomolecules for other diseases. The role of biomolecules in the regenerative process is well established and several peptides have been identified that show promising results in preclinical studies [73-77]. Clinical applications remain limited due to a lack of delivery systems that allow sustained presentation of the bioactive molecules within the site of injury. Rationally designed nanomedicines have the potential to overcome obstacles to tissue regeneration by allowing presentation of bioactive molecules for longer periods while guiding the regenerative processes. Thus combining a delivery system that increases drug half-life of one or more bioactive molecules within the site of injury will improve regeneration of nerve tissue following spinal cord injury (SCI). We have recently developed and tested a novel drug delivery system based on previously published pH sensitive cell membrane spanning peptides [75]. Further we incorporate the laminin-derived bioactive sequence, isoleucine-lysine-valine-alanine-valine (IKVAV), because along with its backbone peptide amphiphile (PA) it has been shown to reduce astrogliosis, reduce neuron cell death, increase the number of oligodendrocytes, promote regeneration of both descending motor fibers and ascending sensory fibers, and improve behavioral function [78]. In culture, bone marrow stromal cells not only proliferate and adhere well, but will also yield more neurons when induced to differentiate into neurocytes on IKVAV peptide nanofiber gel [79]. Simple IKVAV peptide, laminin or control PA which do not

contain neuroactive biotopes have not been able to suppress astrocytic differentiation [66, 78]. Therefore, we hypothesized the biological effect of our IKVAV-peptide is different to previous reports of the method of action of the IKVAV motif alone, it may promote neuron regeneration following SCI to improve neurological outcome, and stimulate neuron regeneration [78]. In preliminary experiments, the non-neurotrophic portion of the peptide (without IKVAV) will adhere to tissue and remain at the site of injection with a long half-life of more than 24 hours. We hypothesized the IKVAV-peptide combination will allow sustained presentation of the IKVAV motif within the site of injury of SCI without requiring additional extraneous chemicals or constructs such as a hydrogel, it would stimulate increased neuron regeneration, and reduced glial scarring leading to improved function in mice with SCI.

Loss of the neuronal Nissl bodies has been shown five minutes after a weight drop SCI in rodents. The electron micrographs of neurons in gray matter have also shown fragmented nucleus and condensed bodies, along with shrunken cell bodies [80]. In addition, following incomplete SCI, atrophy of skeletal muscle occurs and it is replaced by intramuscular fat [81, 82]. These markers of spinal cord deterioration following SCI (glial scar, astrocytes, Nissl bodies, fragmented nuclei, condensed bodies, shrunken cell bodies, and muscle bundle atrophy) were used in this report to determine the effect of IKVAV attached to a membrane spanning peptide administered topically to the injured spinal cord in mice.

The model chosen for determining the effect of this novel Nano peptide was the clip compression injury model. All existing models of SCI in mice produce injury, but none of them could produce an initial impact plus persisting compression, other than the graded model of clip compressive SCI in the mouse used here [78]. Mortality reported to be less than 10% during the injury process and the 0% incidence of mortality during the post-injury neurological assessment period were also considered advantages of this novel model [77].

Self-assembling peptide-based three-dimensional biomaterials are being developed for therapeutic applications that include cell adhesion, proliferation, and differentiation [66, 79, 83]. These materials have some appealing properties for application in regenerative medicine because they mimic the structure of the ECM, are reabsorbable, allow biofunctionalization, and can be injected directly into the lesion [84]. In our study, the transmembrane-spanning peptide presents the neurotrophic biotope IKVAV in the ECM without extraneous materials, such as gelatin or nanofibers, in order to retain the IKVAV signal at the site of injury for a prolonged period to sustain the signal for the neurons to regenerate and to prevent scar formation.

Materials and Methods

Animal care and mouse spinal cord injury

All animal procedures were undertaken in accordance with the Public Health Service Policy on Human Care and Use of Laboratory Animals. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Oregon

State University. Thirty healthy skeletally mature Female Balb-c mice (10 weeks of age) were divided into five groups of 6 animals each with a nonsurgical group of normal mice and surgical treatment groups including: vehicle (mannitol 0.5mg/ml), control (IKVAV 0.5mg/ml), control (peptide, AELLLELELLELELLL, 0.5mg/ml), and treatment (IKVAV-peptide, AELLLELELLELELLLAIKVAV, 0.5mg/ml). All peptides were synthesized with 95% purity. We anticipated needing six mice per treatment group based on analysis of preliminary data; however, this number was increased to account for animal loss, unrelated to treatment. Animals were anesthetized with isoflurane inhalant (4-5% induction, 1-2% maintenance). After laminectomy, at the level of T12 vertebral segment, the spinal cord was compressed dorsoventrally by the extradural application of a 24 g modified aneurysm clip for 1 minute [85]. Following spinal cord injury, the incision was closed using metal staples (Autosuture, Norwalk, CT). Postoperatively, animals were kept in cages placed on a circulating water pad (80 °F) to prevent hypothermia and 1.0 milliliter injection of saline was administered once daily subcutaneously (s.c.) to prevent dehydration. Blocked bladders were manually emptied (by finger pressing the bladder over the pelvic region) two to three times daily throughout the study. We used phenoxybenzamine (5-10 mg/kg, orally) to address urine retention and bladder distention. Phenoxybenzamine is used to reduce internal urethral sphincter tone. It may also be used for urethral spasm secondary to bacterial urethritis [86]. Buprenorphine was given (0.05 mg/kg, s.c., twice daily) for analgesia in the event that the animal showed signs of discomfort (such as chewing, anorexia, and other signs of

pain). Enrofloxacin was administered once daily in the event of hematuria or self-mutilation (5mg/kg,) orally for 5 to 7 days. Mice that exhibited any hind limb movement less than 24 hours following the injury were excluded from the study.

Peptide injection

IKVAV-peptide (0.5 mg/ml, 1% Phosphate Buffered Saline, 2.5% mannitol solution) or other treatments including control peptides at equal molar concentrations, were administered 24 hours after injury using tuberculin syringes with a fine (25 gauge) needle. Under isoflurane anesthesia, the skin sutures were removed and the site of injury (subcutaneous portion) was exposed. A micropipette was inserted just under the granuloma formed at the site of injury dorsally on the spinal cord, and 10 μl of the IKVAV-peptide solution (approximately 5 μg total) or other treatments were applied to the injury site. The needle was carefully withdrawn and the wound was closed. For all experiments, the investigators were kept blind to the identity of the treatment groups.

Basso, Beattie, and Bresnahan open-field locomotion score

To assess behavioral recovery from SCI, the Basso, Beattie, and Bresnahan (BBB) open-field locomotion score was observed using the BBB Locomotor Rating Scale [87, 88]. For examination, the mice were placed individually in an open field on a non-slippery surface. The 22-point (0–21) BBB scale was used to assess hind limb locomotor recovery including joint movements, stepping ability, coordination, and trunk stability. A score of 21 indicates unimpaired locomotion as observed in

uninjured mice whereas a score of 0 indicates complete absence of voluntary movement. The tests were performed by two examiners who were blinded to the animal's treatments. BBB tests were performed every day after injury till the end of the study (28 days).

Animal tissue acquisition and preparation

On day 28, animals were euthanized with an overdose intraperitoneal pentobarbital (beuthanasia-D , Schering-Plough Animal Health Corp., Union, NJ) 100mg/kg (0.2 ml/mouse) and the spinal cords were removed after complete laminectomy at the site of the injury and immediately placed in 2% buffered formalin. The spinal cords were kept in the refrigerator overnight and then transferred to the electron microscopic laboratory for routine tissue processing. Semi-thin sections (0.5μm) were cut using a Sorvall MT2-b (Norwalk Connecticut, USA) microtome for light microscopic observations and then ultrathin sections (60 nm) were cut with a diamond knife and put on 100 mesh copper grids for transmission electron microscopic observation using Philips CM/12 STEM (Eindhoven, Netherlands).

Muscle preparation

Hamstring and quadriceps muscles were dissected from all groups and fixed in 2% buffered formalin for 24 hours. Segments were embedded in paraffin and processed routinely for light microscopic evaluation of 5 μm sections stained with hematoxylin and eosin.

Neuron and muscle bundle size quantification

The computer software ImageJ (Wayne Rasband, National Institutes of Health (NIH), USA) was used to measure the neuron and muscle size. We used micrometer (μm) as a unit for measuring the surface of each neuron and each muscle bundle.

Protoplasmic astrocyte quantification

Astrocytes were counted in 3 fields of each semi-thin section for each mouse at 400 magnification using calibrated ocular lens with Lattice lines.

Statistical analysis

Two-way ANOVA and general linear model were used to analyze the data. Differences in BBB scores, body weight between group means, and protoplasmic astrocytes numbers were analyzed using two-way ANOVA. Neuron and muscle size means were identified using linear model test and a significance level of $p < 0.05$. Statistical analysis was carried out using the open source statistical programming arrangement 'R' [89].

Results

To assess the functional recovery of the spinal cord and measure the basis of the functional recovery following injury, several outcome parameters were used including BBB score, histological and electron microscopy. At 4 weeks after SCI, IKVAV-peptide mice displayed a significant improvement in neurological status ($p<0.05$), whereas none of the other treatment groups were significantly altered from

their immediate postoperative status (Figure 2.1, Supplementary Table 2.1). Large variation in the mannitol vehicle treated group resulted in no significant difference between it and any of the other groups ($p>0.05$).

Effect of the IKVAV-peptide on Protoplasmic Astrocytes

As seen in Figure 1, IKVAV-peptide significantly improved neurological recovery following acute SCI, with improvements in motor function evident as early as 4 weeks following treatment. At 4 weeks following SCI, ocular counting of light-microscopic sections of injured spinal cords identified increased numbers of protoplasmic astrocytes in the IKVAV-peptide treatment group compared to IKVAV, normal and mannitol groups ($p<0.001$) (Figure 2.2). However, IKVAV-peptide was not significantly different from the peptide treated group ($p>0.05$) (Supplementary Table 2.2).

Effect of the IKVAV-peptide on Neuron Size and Neuron Cell Death

Injury-induced apoptosis can be detected from hours up to days following injury and may contribute to neurological dysfunction [90]. We used ImageJ software to measure the size of the neurons in each of the treatment groups to determine whether the injection of the IKVAV-peptide could support cellular regeneration of the motor neurons. The images of all sections were taken using a light microscope equipped with a digital camera (DFC280 Model, Leica Microsystems Ltd) [91], with 400X magnification. There was a significant increase in the size of the neurons of the gray matter in the IKVAV-Peptide mice compared to the other four groups ($p<0.05$)

(Figure 2.3) (Supplementary Table 2.3). Neuron reactivation was seen in the gray matter of the spinal cord at the site of injury. Neuron degeneration and death were detected using light microscope (LM) and transmission electron microscope (TEM) micrographs. The characteristics of neuronal death were examined, included apoptosis and necrosis. These included shrunken cell bodies with intact cell membranes, chromatolysis evidence such as dispersed Nissl's substances, and finally a loss of these cellular organelles throughout the cytoplasm along with loosely dispersed nuclear chromatin. Few expanded empty spaces were also noticed between the neuron cell bodies in IKVAV, peptide, and mannitol mice. Using LM and TEM images we observed 1.4 to 2.4-fold larger neurons in the IKVAV-peptide group (Figure 2.3, panel A1-2).

Effect of the IKVAV-peptide on Muscle Size

Each section was photographed using digital camera connected to the light microscope, with 400X magnification. To measure the muscle bundles we used ImageJ software to assess the degree to which SCI with and without treatment can affect muscle morphology. The results of this measurement indicated that the size of muscle bundles in IKVAV-peptide treated group was significantly increased compare with IKVAV and peptide groups ($p<0.05$) but not with normal and mannitol treated mice ($p>0.05$) (Figure 2.4, Supplementary Table 2.4). Control treated groups showed identified atrophied muscle bundles with considerable amount of muscular fat (Figure 2.4, C-D).

Effect of the IKVAV-peptide on body weight

The body weight was measured for each mouse daily, using a digital scale. Significant differences in the change in body weight was identified only between IKVAV-peptide and mannitol mice ($p<0.05$, 19.9 ± 4.1 versus 13.2 ± 2.0, resp.).

Discussion

The pathophysiology of acute SCI involves primary and secondary mechanisms of injury [17, 25, 29, 92]. These mechanisms involve the initial mechanical damage leading to secondary injury processes that contribute to further tissue loss, functional impairment, and lesion size increase [21, 90]. Secondary injury involves the apoptotic and necrotic death of neurons and glial cells [93, 94]. The microscopic evaluation of neuron death within the lesion site in this study is consistent with other studies, which have described similar amounts of apoptosis and necrotic death after SCI [94-99]. Although SCI leads to neuronal death, we have seen small numbers of surviving neurons with normal intact structure within the lesion site, which is consistent with other studies using the clamp model [100]. In the present study, IKVAV-peptide treated mice revealed the largest number and size of active neurons compared with all other groups ($p<0.05$), thus indicating that the IKVAV-peptide induced an progressive active phase in these neurons to regain their normal function. However, in the other groups, reduction of neuron size characterized with shrunken cell cytoplasm may have represented necrotic and apoptotic phases of SCI, which is in agreement with other reports at 28 days post-injury [99].

The SCI was induced with the clamp crush method which is a technique relevant and similar to clinical SCI in humans [101]. In order to mimic clinical delays in definitive treatment while the patient is stabilized following SCI, we also chose to administer treatment approximately 24 hours following surgery. Recovery was incomplete 28 days following treatment, however, IKVAV-peptide treatment promoted significant functional improvement that can be attributed to the observed differences in neuronal cell death and astrocyte function. Although reactive astrocytes may form a glial scar that can, in some cases, prevent axonal regeneration, they also have a crucial role in wound healing and functional recovery following SCI. Emergence and migration of reactive astrocytes have a prominent role in the repair of injured tissue and the restoration of motor function in the subacute phase of healing neuronal tissue (before completion of the glial scar) [102]. Loss of astrocyte activities during the cellular response to SCI can lead to gross functional deficits and failure of functional recovery and suggest that reactive astrocytes not only protect tissue but also preserve function after SCI [15, 103].

In this experiment, IKVAV-peptide treatment improved neurological function and resulted in an increase in the number of astrocytes. Our results support the positive role of astrocytes in promoting neuron regeneration across the site of injury. Interestingly, the peptide-treated group had a similar increase in the number of astrocytes at the site of injury compared to the IKVAV-peptide group. However, neurological function was not improved in this group suggesting that IKVAV must be present on the peptide to achieve successful restoration of movement. The reason for

similar numbers of astrocytes between these groups is unknown and further investigation into the mechanism of astrocyte induction with the membrane spanning peptide is warranted. Compelling evidence indicates that formation of a glial scar inhibits axonal regeneration 4 weeks after SCI [104]. In agreement with this finding; we observed functional behavioral scores reached a plateau from week 3 to week 4. Reactive astrocytes divide and slowly migrate into CNS injuries, eventually to fill in the vacant space and most of a glial scar is made up of astrocytes. Whether astrocytes inhibit or promote neuronal growth has been investigated [105]. Multiple models have demonstrated the molecular composition of the glial scar and production of inhibitory molecules by astrocytes are contributing factors for regenerative failure of axons following injury [106-110]. Yet other studies suggest there is a promoting effect of astrocytes on axonal regeneration [102-104, 111]. The in vitro evidence for the ability of astrocytes to promote or inhibit axon regeneration is conflicting. In our study, mice treated with IKVAV and a transmembrane spanning peptide or a transmembrane spanning peptide alone, had increases in astrocytes at the site of injury. In contrast to the peptide alone stimulated astrogliosis without neuronal recovery, IKVAV-peptide treatment produced neuronal recovery which occurred along with astrogliosis. Further research is warranted to determine why this difference in functional outcome with a similar increase in astrocytes occurred. However, treatment success with IKVAV-peptide was definitive by our measures.

A major concern for patients following SCI is skeletal muscle atrophy. In rodent models of SCI, skeletal muscle atrophy is associated with smaller muscle

bundles and an increase in intramuscular fat (IMF). Moreover, IMF continues to increase over time in incomplete SCI [82]. IKVAV-peptide treatment prevented muscle atrophy as evinced by increased muscle mass, larger muscle bundle size, and decreased IMF. Further, functional improvement in IKVAV-peptide mice indicates the re-innervation of locomotory muscles.

Our results are significant because none of the mice in our study received physical therapy or exercise of any kind other than being allowed free movement within their cage; yet, mice treated with the IKVAV-peptide did not develop the muscle atrophy and increase in IMF seen in the other treatment groups. Increases in muscle mass are possible even years after injury if the appropriate mode and intensity of exercise are utilized even without spinal cord neuron recovery [111, 112]. Unilateral neuromuscular electrical stimulation (NMES) can evoke hypertrophy in the knee extensor and adjacent skeletal muscle groups and is associated with a reduction in IMF in human beings with chronic SCI. However, it seems rebuilding muscles after SCI needs more time in order to obtain the increased muscle mass that occurs with physical therapy.

Time is an important factor for recovery following injury. We followed the mice for 28 days post-SCI using a similar model as Tysseling et al which reported IKVAV PA-injected group displayed significant behavioral improvement at 5 weeks and thereafter, compare to the control group [64].Surprisingly, treatment with our IKVAV-peptide resulted in significant functional recovery observed approximately

one week after SCI and reached a plateau in improvement of function at week 4. In addition to marked improvement in BBB scores at week 1, several mice displayed self-mutilation. A total of 13 mice chewed on their feet or tails with the majority of the mice in the IKVAV-peptide and mannitol treatment groups (6 mice, mannitol: 4 mice, peptide: 2 mice and IKVAV: 1 mouse). Self-mutilation indicated to us the mice may have been regaining feeling in their extremities. In addition to this, because SCI interrupts neuronal circuits which control the bladder function [39], some of the mice were not able to urinate. Interestingly, in the last week of the study most of the IKVAV-peptide treated mice regained their ability to urinate (4/6) suggesting again re-innervation with restoration of function.

Our observations are similar to kinematic studies of locomotors recovery following SCI in rats with the thoracic clip compression model reporting that during the 6-week post injury period, a significant amount of spontaneous locomotor recovery occurred in 80% of the rats with a return of well-defined locomotor hind limb pattern. However, substantial residual abnormalities persisted up to 6 weeks after SCI including postural deficits [113]. Interestingly, in the last week of the study most of the peptide- IKVAV treated mice regained their ability to urinate (4/6) suggesting again re-enervation with restoration of function.

Further research using greater numbers of animals and following the recovery and neuron regeneration over a longer period of time such as 3 to 6 months is warranted to determine whether the improvements seen in the short term using

IKVAV-peptide are maintained later in the regenerative process. The results suggest a role for cell spanning peptides as a platform nanotechnology for the treatment of injury or disease.

2.6 Acknowledgements

This work was funded by the Department of Defense: W81XWH-04-1-0677 and Oregon State University, College of Veterinary Medicine pilot project program.

Figure legends

Figure 2.1. IKVAV-peptide mice had a significant change in neurological status by 28 days following spinal cord compression injury whereas none of the other treatment groups were significantly altered. There is a large amount of variation in the mannitol vehicle treated group and this resulted in no significant difference between this group and any of the other groups ($p>0.05$). IKVAV-peptide group has the highest improvement in mean BBB score compared to all other groups measured by one way ANOVA ($p=0.0067$) with Dunn's multiple comparison post-test. Standard error (SE) has been shown to describe the mean accurately.

Figure 2.2. The number of protoplasmic astrocytes at the injury site in IKVAV-peptide treated mice is significantly increased compared to all other groups except the peptide group ($p<0.001$). IKVAV-peptide box plot shows normal distribution of protoplasmic astrocyte means compare to all other groups.

Figure 2.3. Mean neuron size was significantly larger in the IKVAV-peptide group compared to all other groups 4 weeks after SCI ($p<0.05$) and exceed the mean size of neurons of non-surgical controls. The pictures presented in **A-D** shows the comparison between different groups at light (**1**) and electron microscope (**2**) level. In IKVAV-peptide group (**A**), the activated large neurons are obvious and actually larger compared to Normal (**B**), however, in control groups IKVAV (**C**), Peptide (**D**), and Mannitol (**E**) the neurons are shrunken and in some cases pyknotic.

Figure 2.4. There is a significant increase in the means of IKVAV-peptide (**A**) muscle bundle size compared to IKVAV (**C**) and Peptide (**D**) ($p<0.007$) but not to Normal (**B**) and Mannitol (**E**) groups. The pictures presented in **A-D** shows the comparative differences between representative groups.

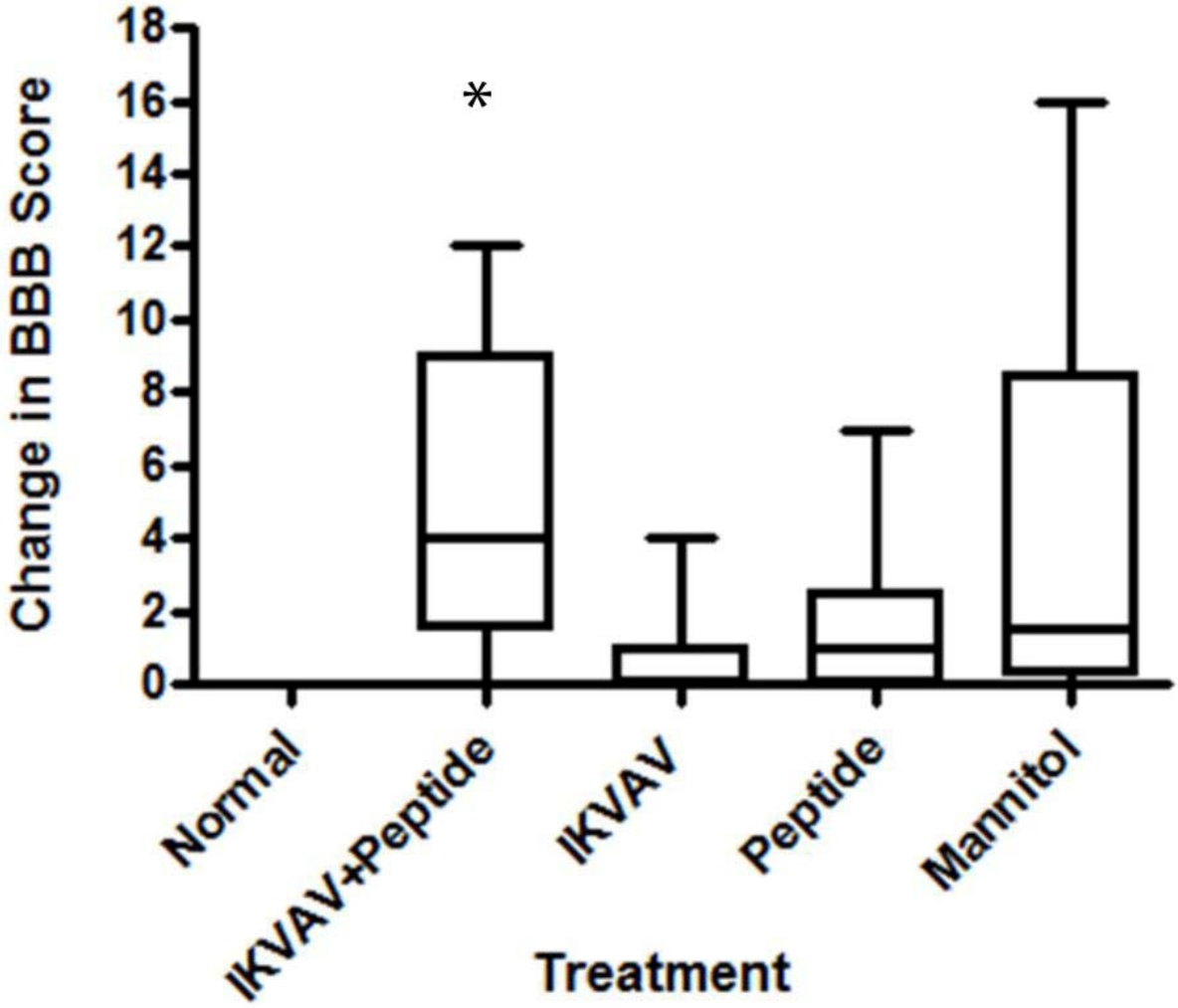
Change in BBB Score
18
16
14
12
10
8
6
4
2
0
*
Normal
IKVAV+Peptide
IKVAV
Peptide
Mannitol
Treatment

Figure 2.1

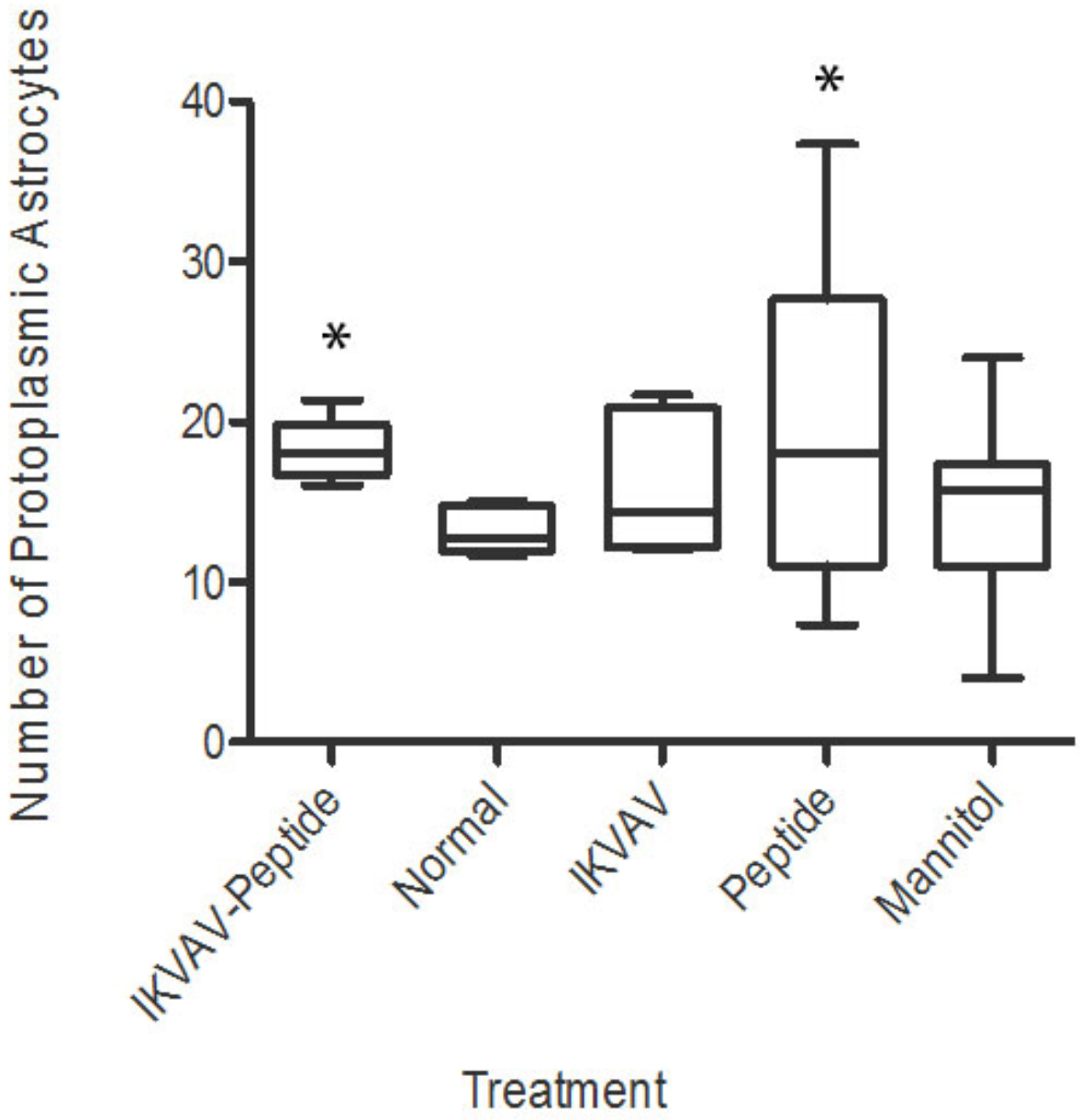
Number of Protoplasmic Astrocytes
40
30
20
10
0
*
*
IKVAV-Peptide
Normal
IKVAV
Peptide
Mannitol
Treatment

Figure 2.2

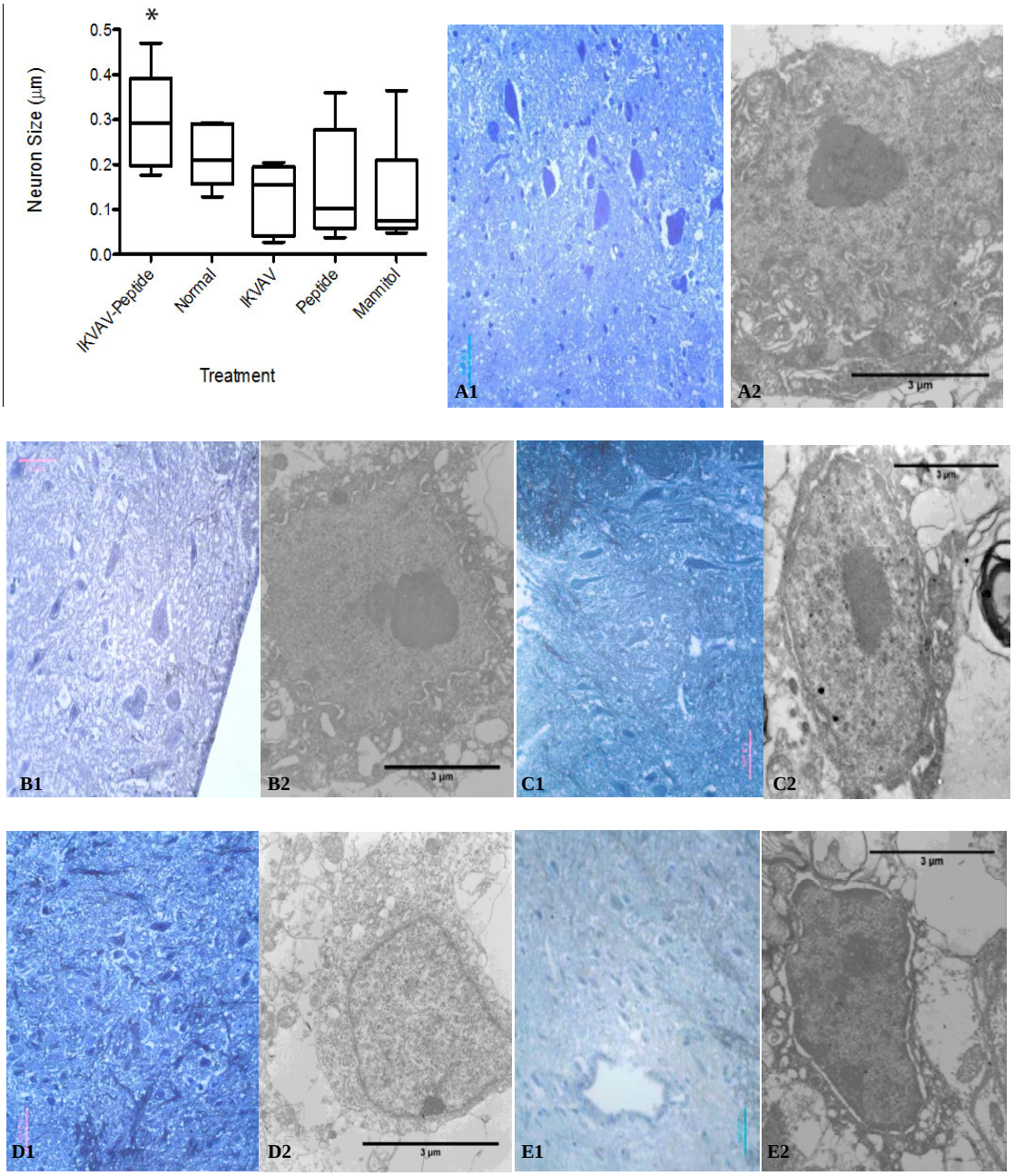
Neuron Size (μm)
0.5
0.4
0.3
0.2
0.1
0.0
*
IKVAV-Peptide
Normal
IKVAV
Peptide
Mannitol
Treatment
A1
A2
3 μm
B1
B2
3 μm
C1
C2
3 μm
D1
D2
3 μm
E1
E2
3 μm

Figure 2.3

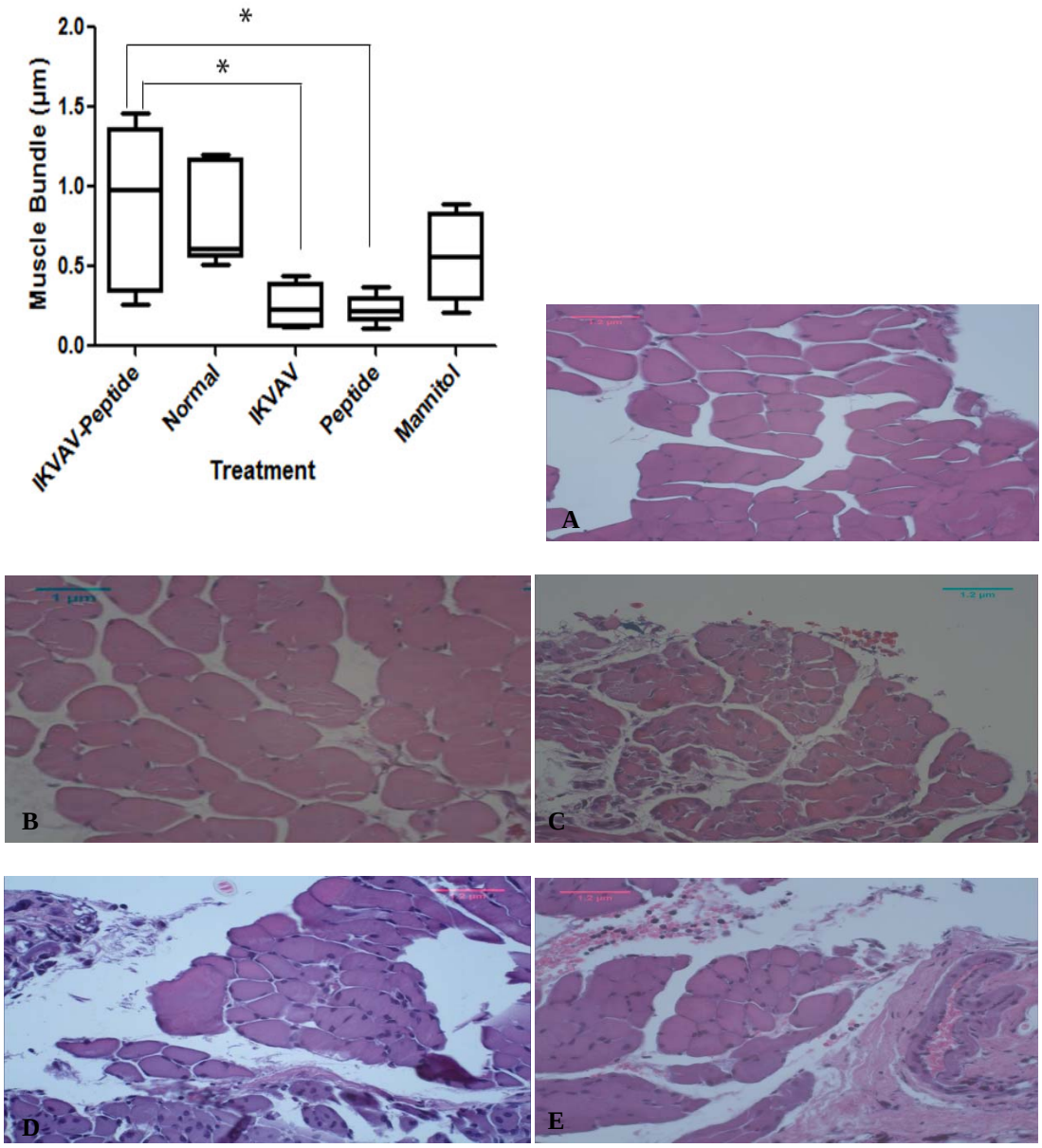
*
*
2.0
1.5
1.0
0.5
0.0
Muscle Bundle (µm)
IKVAV-Peptide
Normal
IKVAV
Peptide
Mannitol
Treatment
A
B
C
D
E

Figure 2.4

Group	Mean ± Standard Error	Minimum	Median	Maximum
Normal	0	0	0	0
IKVAV-peptide	5.4 ± 1.4	0	4	12
Mannitol	4.2 ± 2.04	0	1.5	16
Peptide	1.7 ± 1.09	0	1	7
IKVAV	1 ± 0.54	0	1	4

Table 2.1: Change in BBB scores over time

Group	Mean ± Standard Error	Minimum	Median	Maximum
Normal	13.1 ± 0.57	11.7	12.7	15
IKVAV-peptide	18.2 ± 0.88	16	18	21.3
Mannitol	14.2 ± 2.38	4	15.7	24
Peptide	19.1 ± 4.96	7.3	18	37.3
IKVAV	16.1 ± 2	12	14.3	21.7

Table 2.2: Protoplasmic astrocytes

Group	Mean ± Standard Error	Minimum	Median	Maximum
Normal	0.2 ± 0.02	0.1	0.2	0.3
IKVAV-peptide	0.3 ± 0.05	0.2	0.3	0.5
Mannitol	0.1 ± 0.04	0.05	0.08	0.4
Peptide	0.2 ± 0.06	0.04	0.1	0.4
IKVAV	0.1 ± 0.03	0.03	0.1	0.2

Table 2.3: Neuron size

Group	Mean ± Standard Error	Minimum	Median	Maximum
Normal	0.8 ± 0.15	0.5	0.6	1.2
IKVAV-peptide	0.9 ± 0.23	0.3	1	1.5
Mannitol	0.6 ± 0.12	0.2	0.6	0.9
Peptide	0.2 ± 0.04	0.1	0.2	0.4
IKVAV	0.2 ± 0.06	0.1	0.2	0.4

Table 2.4: Muscle size

Chapter III

IKVAV linked cell membrane penetrating peptide treatment induces neuronal reactivation following spinal cord injury

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Abstract

A transmembrane-spanning peptide linked to the IKVAV peptide known to have a long half-life and the peptide motif, isoleucine-lysine-valine-alanine-valine (IKVAV) was used to evaluate neuronal effects on recovery from spinal cord injury (SCI) in a vascular clamp model of injury in mice. The IKVAV-peptide protein was applied directly to the spinal cord at the site of injury 24 hours following post-trauma. Because the membrane spanning portion of the peptide adheres to tissue upon injection with a half-life of more than 24 hours, we hypothesized that the bioactive IKVAV sequence will provide a sustained regenerative signal at the sight of injury. Light and electron microscopy of spinal cord sections were used to determine the effect of mannitol, IKVAV-peptide, peptide alone, and IKVAV alone applied directly to the spinal cord at the site of injury compared to mice not receiving SCI or treatment. Treatment with IKVAV-peptide following SCI resulted in an increased number of protoplasmic astrocytes and large active motor neurons crossing the site of the SCI and regeneration of muscle bundles in peripheral skeletal muscle in addition to improvement in neurological function.

Keywords: IKVAV-peptide, neurons, protoplasmic astrocytes, muscle bundles, light micrographs, electron micrographs.

Introduction

Spinal cord injury is a debilitating condition that stimulates complex cellular and molecular interactions in the spinal cord at the site of injury and results in altered cellular morphology in an attempt to repair the initial damage. These interactions include contributions from inflammatory cell activation, reactive astrocytes and the production of both growth

promoting and inhibitory extracellular molecules [114]. The use of nanostructures in medicine is an emerging field with the potential to enhance cellular recovery from trauma. To assess the feasibility of using a peptide based nanostructure to facilitate regeneration of damaged spinal tissue, it is necessary to determine the cellular responses within the lesion at the site of injury. Biological analysis of SCI in mouse models has pointed to both necrotic and apoptotic mechanisms of cell death after injury [115]. Electron micrographs of neurons in gray matter following SCI have shown fragmented nuclei and condensed bodies along with shrunken cell bodies. Neuronal and glial apoptosis following SCI can contribute to neurological dysfunction, paresis and paralysis [99] . Spinal cord injury also results in glial reaction which recruits microglia, oligodendrocyte precursors, meningeal cells, astrocytes and stem cells. Most of these cell types produce molecules that have been shown to be inhibitory to axon regeneration.

Activated astrocytes after SCI exhibit different morphology, and the morphological characteristics are distinct at different stages. Activated astrocytes migrate and eventually form a glial scar at the site of the injury which inhibits axon regeneration and recovery of normal function. The glial scar that forms following spinal injury is an evolving structure, with different cells arriving and participating at different stages, to create a final structure that is predominantly astrocytic. This astrocyte response to injury is referred to as reactive gliosis (more glia) but in fact, in most instances of SCI, the actual amount of glial cell division is relatively small and confined to the immediate penumbra surrounding the lesion core [104, 116-119]. Astrocytes form the majority of the scar and may be responsible for inhibition of neuron regeneration across the site of injury.

The laminins are a family of glycoproteins that provide an integral part of the structural scaffolding of basement membranes in almost every animal tissue. Each laminin is a heterotrimer assembled from α, β, and γ chain subunits. Laminin is secreted and incorporated into cell-associated extracellular matrices. The laminins can self-assemble, bind to other matrix macromolecules, and have unique and shared cell interactions mediated by integrins, dystroglycan, and other receptors. Through these interactions, laminins critically contribute to cell differentiation, cell shape and movement, maintenance of tissue phenotypes, and promotion of tissue survival. Laminin 1 is one of the isoforms of laminin which consists of three chains, α1, β1 and γ1. IKVAV is an active sequence of laminin-1 which is located in the C-terminal end of the long arm of the α1 chain which promotes cell adhesion, neurite outgrowth, angiogenesis, collagenase IV production, and tumor growth [60, 61, 120-122]. In a recent study [64], it has been shown that treatment with IKVAV PA (peptide amphiphile) following SCI, reduced astrogliosis and subsequent glial scar as well as cell death, cell death. IKVAV also increased the number of oligodendroglia, promoted regeneration of descending motor fibers and ascending sensory fibers and resulted in significant behavioral improvement in mice.

Skeletal muscle atrophy results from denervation and consequently disuse and immobilization in both complete and incomplete SCI. Within weeks post-injury, skeletal muscle cross-sectional area (CSA) can be as low as 30% to 50% compared with un-injured controls. Moreover, skeletal muscle atrophy has been associated with increasing infiltration of intramuscular fat (IMF) [81, 82, 123]. Skeletal muscle atrophy is a common adaptation after spinal cord injury (SCI) that results in numerous health-related complications [123]. The detrimental effects of atrophy also include altered body composition [124],cardiovascular disease [125],osteoporosis [126]and increased incidence of metabolic syndrome [127].

Our hypothesis was that, the novel IKVAV-peptide conjugate can be used to enhance the reactivation and regeneration processes at the site of injury following SCI by presenting and sustaining the IKVAV motif within the site of the injury. Therefore, the specific purpose of this study was to evaluate the functional recovery by means of BBB score and histological observations in mice treated with the IKVAV-peptide following SCI in a mouse clip-compression model. We analyzed nerve cells, astrocytes and glial scar formation in the injured spinal cord. In addition, we determined the effect of IKVA-peptide on the rate of muscle atrophy following SCI.

Materials and Methods

Experimental Design

All animal procedures were undertaken in accordance with the Public Health Service Policy on Human Care and Use of Laboratory Animals. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC). Five groups of female Blab-c mice (10 weeks of age) weighing 24-25 grams were used in this study. Animals were anesthetized with isoflurane (4-5% induction, 1-2% maintenance). After hemilaminectomy, all groups underwent T12 spinal cord segment compression dorsoventrally by the application of a 24 g modified aneurysm clip for 1 min [85]. Twenty-four hours after injury, each group received one of the following solutions applied directly to the spinal cord: IKVAV-peptide, IKVAV, peptide and mannitol. Following surgery, the skin was sutured using metal staples (Autosuture, Norwalk, CT). Post –operatively, animals were kept in cages placed on circulating water pad (80◦ F) to maintain body temperature. A 1.0 ml injection of saline was given once daily subcutaneously (s.c.). Distended urinary bladders were manually expressed (by finger pressing the bladder over

the pelvic region) two to three times daily throughout the study. In the event that animal showed signs of discomfort such as chewing, anorexia and other signs of pain, buprenorphine was administered (0.05 mg/kg, s.c., twice daily). Enrofloxacin was administered once daily in the event of hematuria (5mg/kg,) orally for 5 days and, if animals self-maimed, was given orally for at least 7days at this same dose. Mice that exhibited any hind limb movement 24 hours after the injury were excluded from the study. For all experiments, the experimenters were kept blind to the treatment protocol of each group of animals.

Peptide injection

Peptide (1%PBS (Phosphate Buffered Saline), 2.5% mannitol solution) or other treatments were injected 24 hours after injury using sterile micropipette. Under isoflurane anesthesia, the skin autoclips were removed and the injury site was exposed. The micropipette was inserted just under the granuloma formed at the site of injury which contained 10 µl of the diluted peptide solution (5 µg of IKVAV-peptide) or other control solution. At the end of injection the needle was carefully withdrawn and the wound was closed.

Animal tissue acquisition and preparation

On the day 28, animals were euthanized with a 0.2 ml/mouse intraperitoneal Beuthanasia-D [1]and the spinal cords were removed and immediately placed in 2% buffered formalin. The spinal cords were kept at 4° C overnight and transferred to the karnovsky fixative [128] before placing at 4° C for 3 hours. The tissue was rinsed four times in buffer (Cacodylate, 0.1 M, pH 7.2) for 30 minutes each and then incubated in osmium tetroxide (OsO_4) for 1 hour at 25°C. The

[1] Schering-Plough Animal Health Corp., Union, NJ

dehydration procedures were performed using 50% acetone, 70% acetone twice and 100% acetone each for 30 minutes, respectively.

After dehydration, the spinal cords were infiltrated using acetone: spur resin mixture first with a proportion of 3:1 for 30 minutes following by 1:1 and 1:3 each for one hour and then 100% spurs for overnight on a rotating wheel. After 24 hours, each spinal cord was embedded in the flat mound and kept at 63 ^{o}C overnight. Semi-thin sections (0.5µm) were cut using Sorvall MT2-b [2]for light microscopic observations and then (0.06 µm) ultrathin sections were cut with a diamond knife and put on 100 mesh copper grids for transmission electron microscopic study using Philips CM/12 STEM[3].

Muscle preparation

Hamstring and quadriceps muscles were dissected from all groups and fixed in 2% buffered formalin for 24 hours and processed routinely for light microscopic evaluations with sections of 5 microns.

Neuron and muscle size quantification

The computer software ImageJ[4] was used to measure the neuron and muscle size of each mouse using images obtained from the above sections. A micrometer fitted into the microscope was used to calibrate image size, along with magnification strength. We used µm as a unit for measuring the surface area of each neuron and each muscle bundle.

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Protoplasmic astrocytes quantification

Astrocytes were counted in 3 fields of each semi-thin section for each mouse at 400X magnification using calibrated ocular lens with Lattice lines[5].

Statistical Analysis

Two-way ANOVA and generalized linear model were used to analyze the data. Differences in BBB scores, body weight between group means, and protoplasmic astrocytes numbers were analyzed using a two-way ANOVA. Neuron and muscle cross-section means were identified using a linear model test and a significance level of $p < 0.05$. Statistical analysis was carried out using the open source statistical programming arrangement 'R' [89].

Results

Light microscopic observations using semi-thin sections revealed that the main morphological difference between IKVAV-peptide treated mice and the other treatment groups was the size of the motor neurons. IKVAV-peptide treated mice (Figure 3.1, B&C) had neurons larger in cross-sectional size than the normal group (Figure 3.1, A) which did not have SCI or treatment ($p<0.05$). On the other hand, the other three treatment groups (IKVAV alone, peptide alone, mannitol) had smaller neurons with shrunken cell cytoplasm compared to the normal mice ($p>0.05$).

[5] Leica ocular lens, German, Optic Company

Among the three surgical groups (IKVAV alone, peptide alone, mannitol), the peptide group had severely shrunken neurons; however mannitol and IKVAV groups had small neurons that were not as small as the peptide group but still not equal in size to the normal control group (Figure 3.2).

At the electron microscopic level, a survey of ultrathin sections (0.06 μm) revealed that in the IKVAV-peptide group, all the motor neurons were active consisting of active cytoplasmic organelles such as extensive rough endoplasmic reticulum (RER), free ribosomes, developed Golgi complex and mitochondria surrounding a euchromatic nucleus (Figure3.3). These ultrastructural characteristics were similar in appearance to the normal control group (Figure 3.4), however, IKVAV-peptide treated neurons were much larger and active than normal controls confirming observations made at the light microscopic level. In the treated control groups (IKVAV alone, peptide alone, mannitol), motor neurons were non-active with shrunken cytoplasm having dispersed segmented RER and loosely associated nuclear chromatin in their nuclei (Figure 3.5). Particularly in these mice, the nuclear membranes were not folded unlike the normal and IKVAV-peptide treated groups. In addition, the outer nuclear membranes lacked ribosomal attachments in most areas of the three treated control groups (Figure 3.6).

In this experiment, counting the number of protoplasmic astrocytes in each group 4 weeks following SCI and treatment, demonstrated that the IKVAV-peptide treated mice had more protoplasmic astrocytes surrounding the motor neurons compared to the normal untreated, mannitol and IKVAV groups ($P<0.001$). However, the protoplasmic astrocytes were similar in number in both IKVAV-peptide and peptide groups ($P>0.05$). Observations of the proximal hind limb muscles (quadriceps and hamstrings) revealed that in the IKVAV-peptide treated group,

muscle bundles were thicker in comparison to the IKVAV alone- and peptide alone-treated groups ($P<0.05$), but not with mannitol-treated and normal untreated mice muscle ($P>0.05$). In addition, the intramuscular fat seen between bundles occupied less space in IKVAV-peptide group than in the other groups (Figure3.7). Locomotion score scale, Basso, Beattie, Bresnahan (BBB), showed statistically significant functional improvement in the IKVAV-peptide treated group which corresponded with the light and electron microscopic findings ($P<0.0067$). There were some deaths during the study in each of the four treatment groups. Some of the mice were euthanized due to self-mutilation: 5 mice in IKVAV-peptide group, 2 mice in the peptide only group, 3 mice in the mannitol group and 2 mice in the IKVAV only group. Some of the mice died overnight at some time during the first two weeks following SCI and the cause was not identified on necropsy: 1 in the peptide group and 2 in IKVAV group. In addition, one mouse died within 5 minutes of rupture of the bladder while it was manually expressed.

Discussion

The laminin-derived IKVAV sequence was incorporated into peptide amphiphile (PA) in order to enhance neural attachment, migration and neurite outgrowth from inactive neurons following SCI [129].To explore the cellular changes related to this improvement, light and electron microscopic evaluation of neurons, protoplasmic astrocytes and muscle bundles was undertaken. In this study, IKVAV-peptide treatment resulted in neurite reactivation in gray matter, evinced by functional improvement using BBB locomotion score scale and the morphological changes that demonstrated the slow but impressive functional progress following treatment.

Neuronal death is normal during nervous system development but it is an abnormal consequence of brain and spinal cord injury with two distinct forms of cell death, apoptosis and necrosis [130]. Apoptosis and necrosis of neurons in the mice not treated with IKVAV-peptide following spinal cord injury in this study is consistent with previous reports using this mouse model of SCI [90, 93-95, 99, 131, 132]. The reactivation of neurons in IKVAV-peptide treated mice compared to the treatment control groups was evidenced by the significantly increased size of the neurons, abundant organelle presence, and nuclear membrane folding. Previous research has demonstrated that the absence of astrocytes in both white and gray matter following SCI results in unsuccessful axon regeneration and that the presence of immature fibrous astrocytes in the white matter and reactive protoplasmic astrocytes in the gray matter of the spinal cord within the site of injury in the early phase are necessary for axon regeneration [15, 129, 133]. Protoplasmic astrocyte enumeration in the IKVAV-peptide group demonstrated increased number of astrocytes compared to all other groups including the normal control group. Our results support the evidence that, at least in the subacute phase, the existence of astrocytes is necessary for reactivation of neurons and subsequent regeneration of axons; and further, that their absence can markedly exacerbate neuron and glial cell degeneration [102, 103, 134, 135].

Reports of successful spinal cord therapies have correlated neurological functional recovery with reduced muscle atrophy following treatment [123, 130, 136]. Significant functional improvement was observed in IKVAV-peptide treated mice compared to the other treatment groups, along with reduced muscle atrophy (increased muscle bundle mass and decreased intramuscular fat) compared to IKVAV, peptide or mannitol treatment controls. Further investigation of IKVAV-peptide and the mechanism involved in preservation of muscle mass following SCI is warranted.

Conclusions

Investigators were blinded to evaluate the effects of the novel nanostructure IKVAV-peptide and assess its efficacy. Our findings demonstrate that the novel IKVAV-peptide injected at the site of spinal cord injury may improve the functional behavior of neurons and astrocytes at the site of injury which could result in improved outcome in other species including humans. The neurons in IKVAV-peptide group became more active presumably to compensate for deficits following injury as evinced by more functional organelles compared to all groups including neurons found in normal spinal cord tissue. These findings revealed that a peptide that includes a cell spanning moiety and the neurite-stimulating motif IKVAV is a unique structure that has the potential to improve the recovery among patients with SCI. However, these results are preliminary and this research would benefit from larger numbers of animals and longer duration of assessment following treatment to develop better and complete understanding of the applications and limitations of this technology. Therefore, further investigation is warranted to evaluate the full potential of this treatment approach in SCI and other conditions where peptide mediated regeneration is possible.

3.7 Acknowledgements

This work was funded by the Department of Defense: W81XWH-04-1-0677 and Oregon State University, College of Veterinary Medicine pilot project program.

Figure legends

Figure 3.1. Photomicrograph of untreated normal mouse (A) showing a portion of ventral horn of the gray matter. A few relatively large motor neurons with pale nuclei and dark nucleoli can be seen between cellular processes (large arrows). Protoplasmic astrocytes are also evident (small arrow). Photomicrographs of IKVAV-peptide treated mouse (B&C) revealing very large active motor neurons (arrows) surrounded by many nerve fibers (arrows head) in both pictures. Many protoplasmic astrocytes are visible (small arrows). In Figure C a cross section of spinal canal lining by large cuboidal cells can also be seen (C). Toluidine Blue X400.

Figure 3.2.In this photomicrograph (A) of peptide treated control mouse most of the motor neurons are atrophied (arrows). Photomicrograph showing only the gray matter portion of the spinal cord in mannitol treated control mouse (B). All motor neurons are atrophied (arrows) compare with figures 1A &B (normal and IKVAV-peptide). This photomicrograph (C) shows a portion of ventral horn of gray matter between white matter (WM) from IKVAV treated control mouse. Note the atrophied motor neurons (arrows). Toluidine Blue X400.

Figure 3.3. Electron micrograph of IKVAV-peptide treated mouse (A) showing association of protoplasmic astrocyte (PA) with active motor neuron (MN). Some myelinated fibers are also visible (arrow). Uranyl acetate and lead citrate X3400.
Electron micrograph of IKVAV-peptide treated mouse at higher magnification (B). Two active motor neurons can be seen, each with large euchromatic nucleus and prominent electron dense nucleolus. Many cisternae of RER (arrows), active Golgi complexes (G) and mitochondria (*) are also evident throughout the perikaryon. Note the highly folded nuclear membranes in both cells. Compare to untreated normal mouse (Figure 3.4) these motor neurons are much larger. Uranyl acetate and lead citrate X7100.

Figure3.4. Electron micrograph of untreated normal mouse. In this micrograph (A), a protoplasmic astrocyte (PA) can be seen in close association with a motor neuron (MN). Motor neuron has a large euchromatic nucleus with prominent dense nucleolus surrounded by small portion of cytoplasm rich in Nissl substances, RER at arrows and free ribosomes (Circle). A few mitochondria (M) can also be seen. Uranyl acetate and lead citrate X7100.
This electron micrograph (B) shows active motor neuron of untreated normal mouse with extensive Nissl substances (arrows) and mitochondria (M) at higher magnification. Infolding of nuclear membranes is extensive with ribosomes associated outer membrane. Uranyl acetate and lead citrate, X8800.

Figure3.5. In peptide treated control mouse (A) most of the motor neurons are degenerated and atrophied which resulted in presence of a few motor neurons in gray matter (arrow). Uranyl acetate and lead citrate X2650.
Electron micrograph of peptide treated control mouse at higher magnification (B) shows two atrophied motor neurons surrounded by few myelinated nerve fibers. In addition to small perikaryonic portions, dispersed and loosely associated nuclear chromatin are present. Uranyl acetate and lead citrate X5600.

This electron micrograph from peptide treated control mouse at higher magnification shows obviously a shrunken motor neuron with dispersed nuclear chromatin. In all control groups including this control (C), the nuclear membranes are not folded compared with normal and IKVAV-peptide treated groups. The outer nuclear membrane is also lack of ribosomes in most areas (arrows) Uranyl acetate and lead citrate X7100.

Figure 3.6. Electron micrographs of motor neuron in mannitol treated mouse (A) revealing another atrophied neurons. The small cytoplasmic portion is mostly occupied by degenerated mitochondria (M). Uranyl acetate and leas citrate X8800.
Electron micrograph of a shrunken motor neuron of IKVAV treated control mouse (B). Even at this magnification the cellular cytoplasm is not apparent. Myelinated fiber (large arrow) and non-ribosomal outer membrane attachment (small arrow). Uranyl acetate and lead citrate X15000. Note the lack of nuclear membrane infolding in all control groups.

Figure3.7. Comparative photomicrographs showing cross sections of the hind limb muscles in different groups, normal (A), IKVAV-peptide (B), mannitol (C), peptide (D), IKVAV (E) of mice at the same magnification. Note the presence of thicker bundles in IKVAV-peptide group compared to IKVAV and peptide groups. H & E, X400.

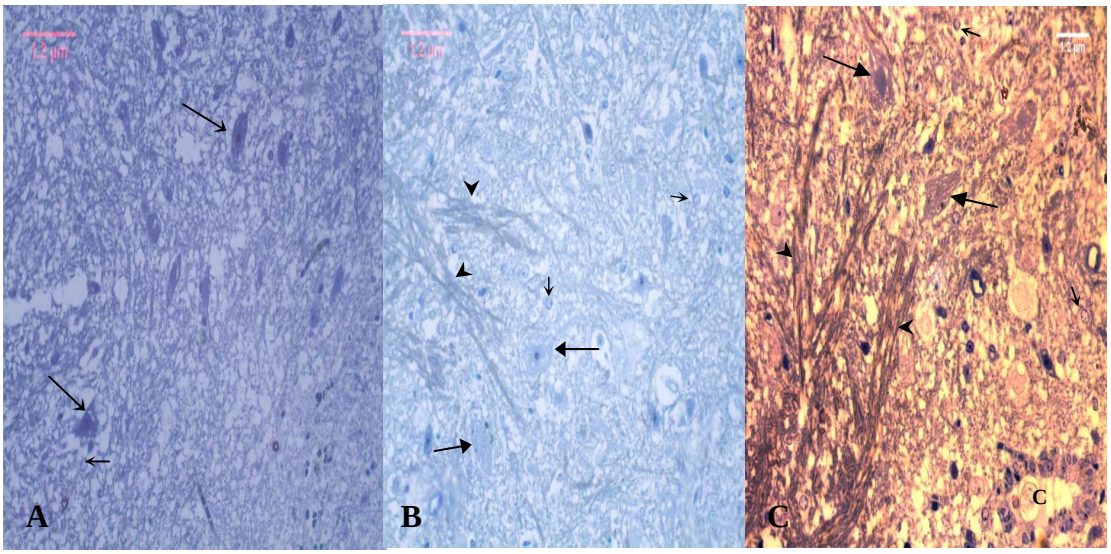

Figure 3.1

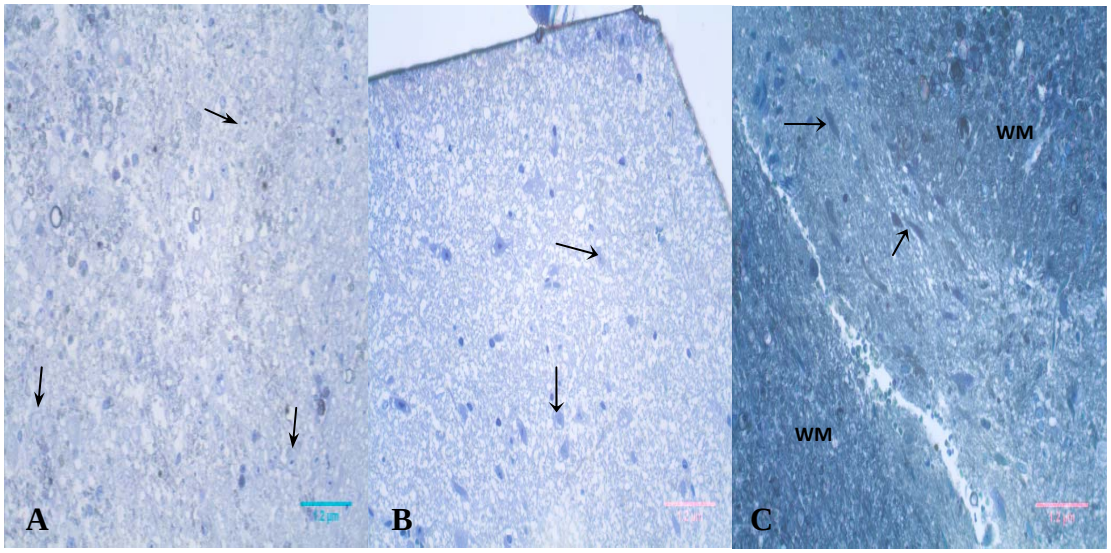

Figure 3.2

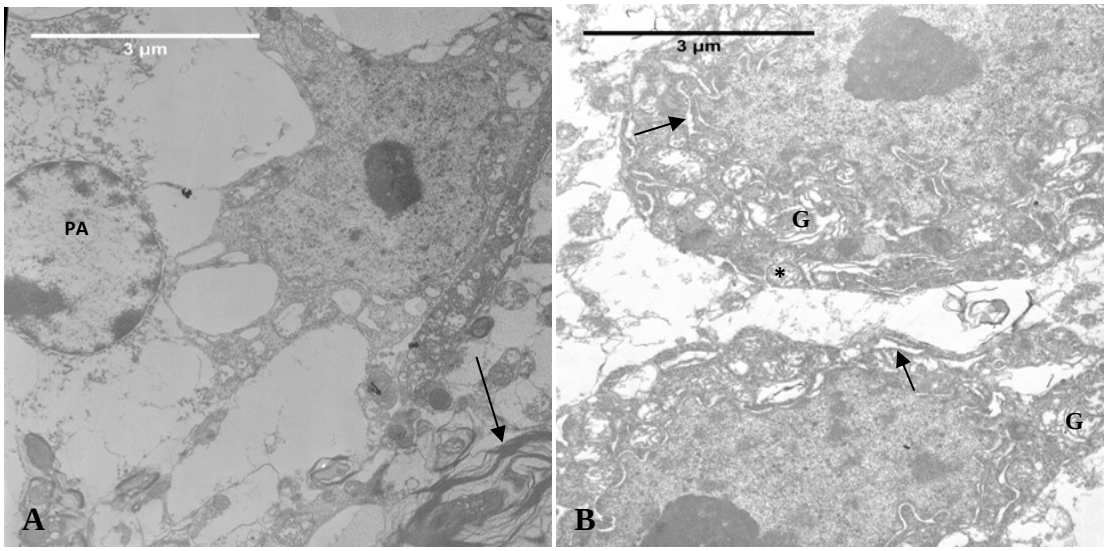

Figure 3.3

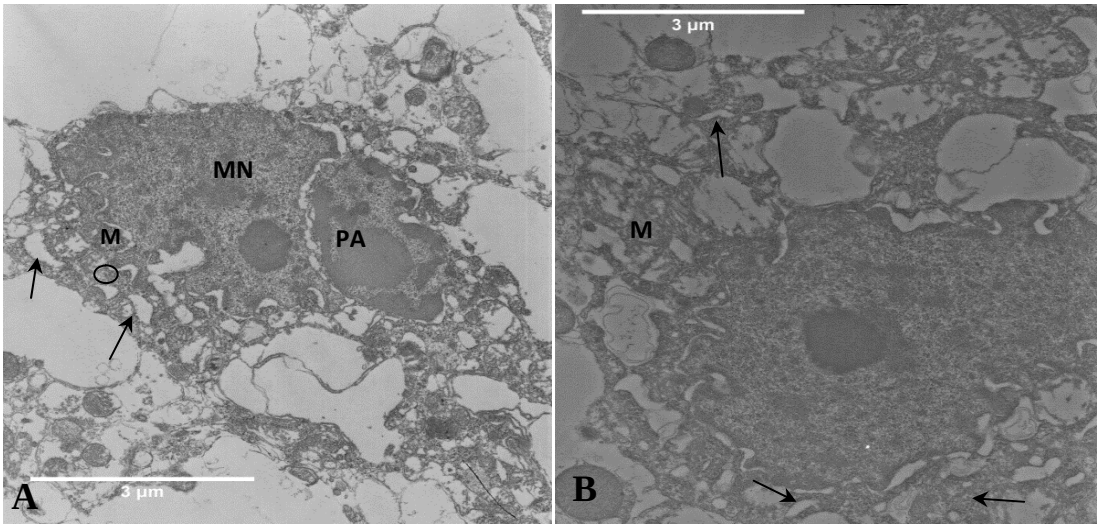

Figure 3.4

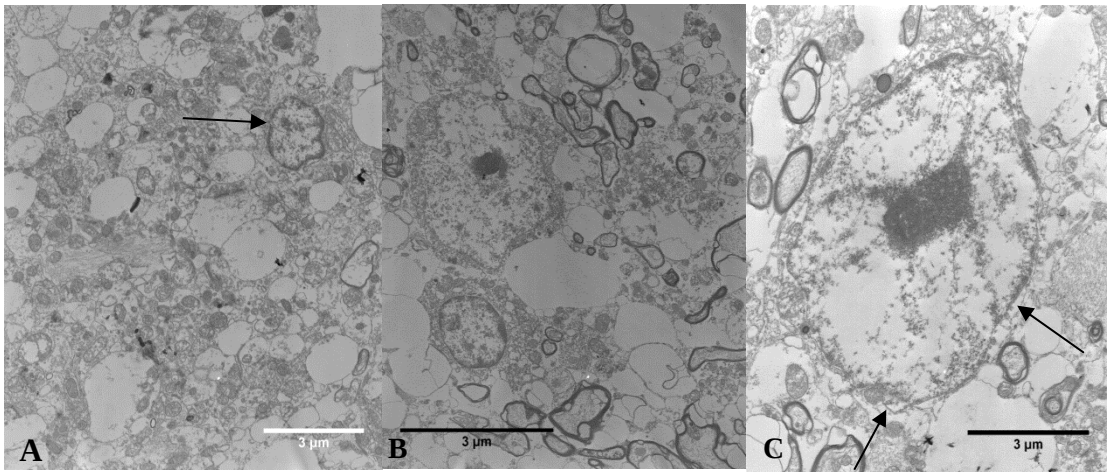

Figure 3.5

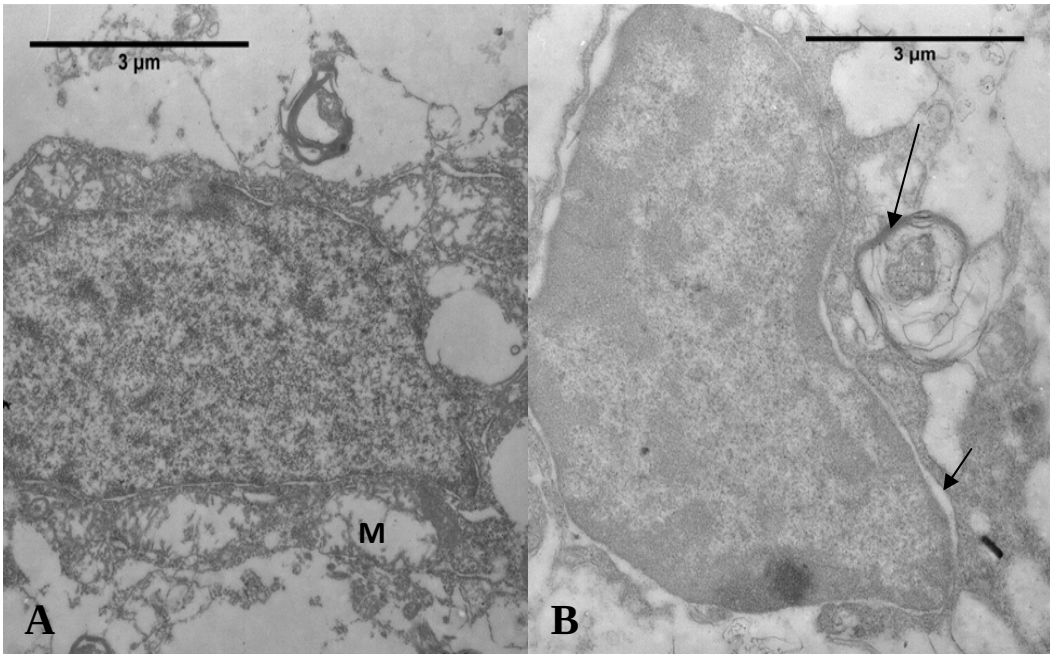

Figure 3.6

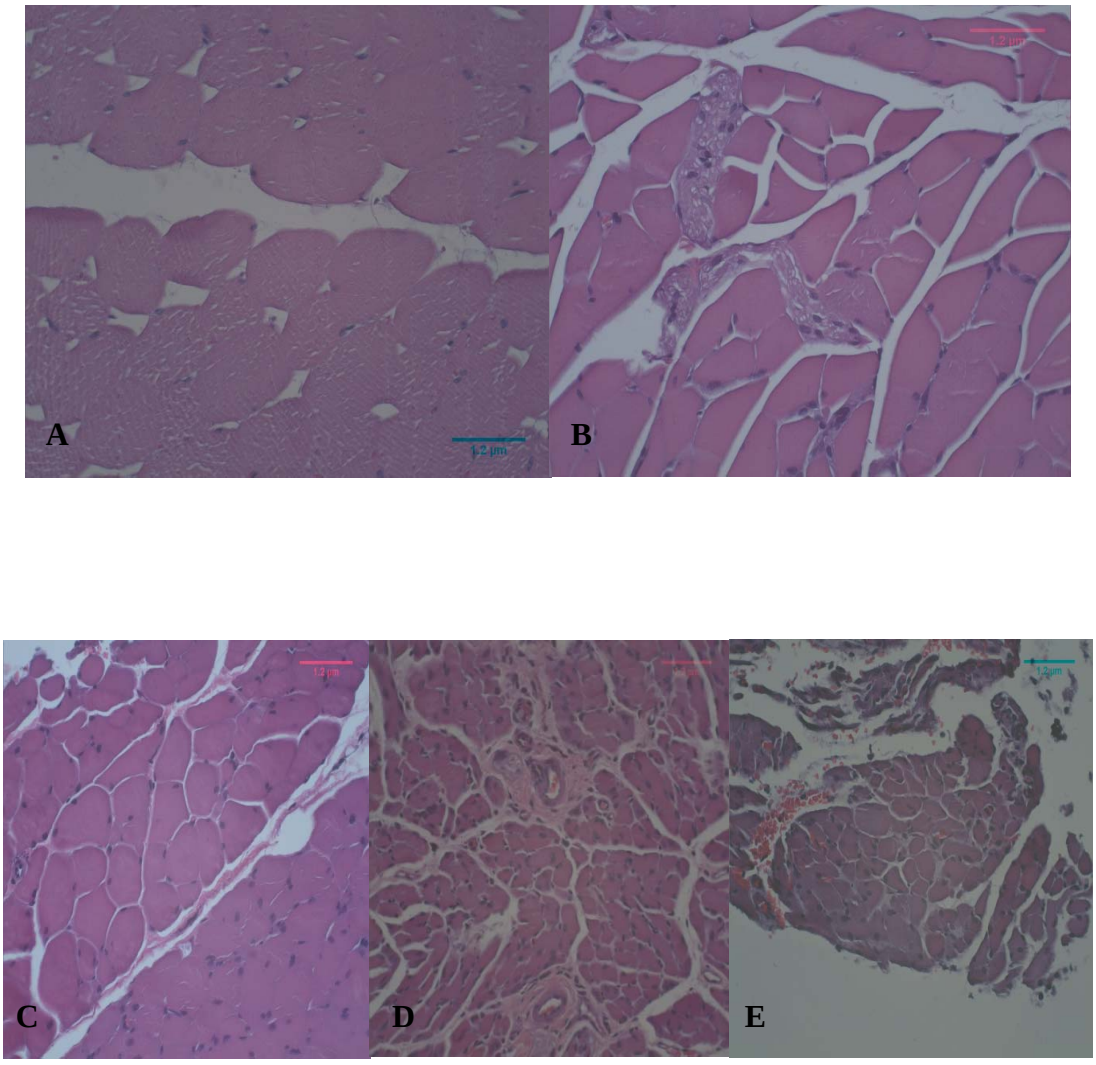

Figure 3.7

Chapter IV

4.1 Summary

The Role of Animal Model

Clip compression injury resembles spinal cord injury caused by pressure to the spinal cord. The spinal cord becomes ischemic and mimics common clinical injuries and outcomes [137]. Investigations of possible cures and of ways to alleviate the hardships of traumatic SCI include those of interventions that attenuate or overcome the secondary injury cascade, enhance the endogenous repair mechanisms, regenerate axons, replace lost cells, and rehabilitate. These investigations have led to the creation of laboratory animal models of the different types of traumatic human SCI and components of the secondary injury cascade. However, no particular model completely addresses all aspects of traumatic SCI. Importantly, as researchers in this area move toward clinical trials to alleviate the hardships of traumatic SCI, there is a need for standardized small and large animal SCI models as well as quantitative behavioral and electrophysiological assessments of their outcomes so that investigators testing various interventions can directly compare their results and correlate them with the molecular, biochemical, and histological alterations [138]. In our experiment mouse model was used which had some limitations. One of the limitations was the small size of the spinal cord segments which was somehow difficult to be handled for histological procedures. These limitations mainly include the mushy characteristic of the spinal texture which was overcome by pouring of fixative over the cord and the small diameter of the spinal segments. From the beginning of the

experiment self-mutilation was one of the main reasons for euthanizing the mice. Although we used a collar to prevent further self-injuries, this particular device was not successful in prevention of self-mutilation. Therefore, larger animal models such as rats or even pigs may provide more data for analysis and may enable improved supportive care during recovery when sensation is returning and the likelihood of self-mutilation is highest.

To compress the spinal cord dorsoventrally we had to perform hemilaminectomy and remove the dorsal lamina of each vertebra at the site of surgery. After 28 days we noticed histologically a bone growth into the spinal cord from the edges of the removed vertebra which completely filled the gray matter with bone cells in some of the mice. One of the ideas to overcome this problem may be to fill the site of the laminectomy with materials such as gelatin or fascia to prevent bone ingrowth.

Due to the mentioned complications and problems particular to this experiment it may be logical to increase the number of mice in each experimental group. In addition, other treatment methods need to be explored such as administration of the IKVAV-peptide intravenously instead of at the site of injury, at various time points following injury, and potentially multiple administrations of the IKVAV-peptide complex to the site of injury. Potential improvement in the response to the treatment may be gained by administering the drug prior to the onset of secondary spinal cord injury such as within 6 hours of the onset of injury. Another consideration may include varying concentrations of the IKVAV-peptide so that an optimal dose and timing as well as frequency of administration could be obtained.

The results of these preliminary experiments indicate that administration of the IKVAV-peptide complex 24 hours following spinal cord injury in an experimental model of

injury in mice results in improved functional outcome, increased neuron regeneration, increased astrocyte concentration at the site of injury and reduced muscle atrophy at 28 days following injury. These findings warrant further investigation of this peptide complex in the treatment of acute spinal cord injury.

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4.3 Vita

ACADEMIC HISTORY

Doctor of Veterinary Medicine Shiraz University, School of Veterinary Medicine, Shiraz, Iran, 2008.

Master of Sciences Oregon State University, School of Veterinary Medicine, Corvallis, OR, USA, 2012.

DVM Thesis: Histochemical, ultrastructural and angiogenesis observations in the heart of male puppy following ligation of left coronary artery and its branches.

M.S Thesis: Morphological and Neurological Outcome in the Short Time Study after Spinal Cord Injury in Mice

SEMINARS and PUBLICATIONS

1- Kazemi S, Mansouri S.H and Tabatabaei A. Observation on ligation of the branch of coronary artery and angiogenesis via surgery in dog. 6th Iranian Symposium of veterinary surgery, Anesthesia and Radiology. Oct 31- Nov2,2006, Mashhad/ Iran.

2- Kazemi, Soheila, Mansouri, Hadi and Tabatabaei, Abotorab. Histomorphological and angiogenesis observations on the heart of the male puppy following ligation of the paraconal artery. J. Appl. Anim. Res. 36(2009), 77-80.

3- Kazemi S.,Mansouri S.H ,and Tabatabaei Naeini A. A study of angiogenesis on dog heart wall. Research Iranian Veterinary Journal Winter 2010; 5(4 (25)):69-72.

4- Soheila Kazemi, DVM, MS, Wendy Baltzer, DVM, PhD, DACVS, John E. Mata, PhD, Karl Schilke, PhD, Hadi Mansouri, PhD. A cell spanning IKVAV expressing peptide for treatment of spinal cord injury. Submitted to the Journal of Nanomedicine.

5- Soheila Kazemi, DVM, MS, Wendy Baltzer, DVM, PhD, DACVS, John E. Mata, PhD, Hadi Mansouri, PhD Karl Schilke, PhD. IKVAV linked cell membrane penetrating peptide treatment induces neuronal reactivation following spinal cord injury**.** Submitted to the Journal of Neuroscience.

6- Poster presentation “The effect of IKVAV- Peptide on spinal cord regeneration following spinal cord injury” Oregon Biosciences Association Conference, Portland, Oregon, October 9-10, 2012.

7- Poster presentation “A cell spanning IKVAV expressing peptide for treatment of spinal cord injury” 2012 CGRB Fall Conference, Corvallis, Oregon, September 21, 2012.

8- Poster presentation “The effect of IKVAV- Peptide on spinal cord regeneration following spinal cord injury” Society for Neuroscience meeting, New Orleans, Louisiana, October 13, 2012.

Laboratory Skills:

1- Tissue processing for light microscopy
2- Tissue processing for electron microscopy
3- Histochemical staining
4- Microtomy
5- Electron microscopy
6- Laboratory animal surgery
7- Scanning electron microscopy
8- Latex vascular injection